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Patents ADP number *(if you know it)*

85712420001

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SUBSTANCES

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SUBSTANCES

The present invention relates to proteinaceous particles, for example phage or ribosome particles, displaying T cell receptors (TCRs).

Background to the Invention

Native TCRs

As is described in, for example, WO 99/60120 TCRs mediate the recognition of specific Major Histocompatibility Complex (MHC)-peptide complexes by T cells and, as such, are essential to the functioning of the cellular arm of the immune system.

Antibodies and TCRs are the only two types of molecules which recognise antigens in a specific manner, and thus the TCR is the only receptor for particular peptide antigens presented in MHC, the alien peptide often being the only sign of an abnormality within a cell. T cell recognition occurs when a T-cell and an antigen presenting cell (APC) are in direct physical contact, and is initiated by ligation of antigen-specific TCRs with pMHC complexes.

The native TCR is a heterodimeric cell surface protein of the immunoglobulin superfamily which is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. TCRs exist in αβ and γδ forms, which are structurally similar but have quite distinct anatomical locations and probably functions. The MHC class I and class II ligands are also immunoglobulin superfamily proteins but are specialised for antigen presentation, with a highly polymorphic peptide binding site which enables them to present a diverse array of short peptide fragments at the APC cell surface.

Two further classes of proteins are known to be capable of functioning as TCR ligands. (1) CD1 antigens are MHC class I-related molecules whose genes are located on a different chromosome from the classical MHC class I and class II antigens. CD1 molecules are capable of presenting peptide and non-peptide (eg lipid, glycolipid)

moieties to T cells in a manner analogous to conventional class I and class II-MHC-pep complexes. See, for example (Barclay et al, (1997)The Leucocyte Antigen Factsbook 2nd Edition, Acadmeic Press) and (Bauer (1997) Eur J Immunol 27 (6) 1366-1373)) (2) Bacterial superantigens are soluble toxins which are capable of binding both class II MHC molecules and a subset of TCRs.(Fraser (1989) Nature 339 221-223) Many superantigens exhibit specificity for one or two Vbeta segments, whereas others exhibit more promiscuous binding. In any event, superantigens are capable of eliciting an enhanced immune response by virtue of their ability to stimulate subsets of T cells in a polyclonal fashion.

The extracellular portion of native heterodimeric αβ and γδ TCRs consist of two polypeptides each of which has a membrane-proximal constant domain, and a membrane-distal variable domain. Each of the constant and variable domains includes an intra-chain disulfide bond. The variable domains contain the highly polymorphic loops analogous to the complementarity determining regions (CDRs) of antibodies. CDR3 of αβ TCRs interact with the peptide presented by MHC, and CDRs 1 and 2 of αβ TCRs interact with the peptide and the MHC. The diversity of TCR sequences is generated via somatic rearrangement of linked variable (V), diversity (D), joining (J), and constant genes

Functional α and γ chain polypeptides are formed by rearranged V-J-C regions, whereas β and δ chains consist of V-D-J-C regions. The extracellular constant domain has a membrane proximal region and an immunoglobulin region. There are single α and δ chain constant domains, known as TRAC and TRDC respectively. The β chain constant domain is composed of one of two different β constant domains, known as TRBC1 and TRBC2 (IMGT nomenclature). There are four amino acid changes between these β constant domains, three of which are within the domains used to produce the single-chain TCRs displayed on phage particles of the present invention. These changes are all within exon 1 of TRBC1 and TRBC2: N_4K_5->K_4N_5 and E_{37}=>Y (IMGT numbering, differences TRBC1->TRBC2), the final amino acid change

between the two TCR β chain constant regions being in exon 3 of TRBC1 and TRBC2: V_1->E. The constant γ domain is composed of one of either TRGC1, TRGC2(2x) or TRGC2(3x). The two TRGC2 constant domains differ only in the number of copies of the amino acids encoded by exon 2 of this gene that are present.

The extent of each of the TCR extracellular domains is somewhat variable. However, a person skilled in the art can readily determine the position of the domain boundaries using a reference such as The T Cell Receptor Facts Book, Lefranc & Lefranc, Publ. Academic Press 2001.

Recombinant TCRs

The production of recombinant TCRs is beneficial as these provide soluble TCR analogues suitable for the following purposes:

- Studying the TCR / ligand interactions (e.g. pMHC for αβ TCRs)
- Screening for inhibitors of TCR-associated interactions
- Providing the basis for potential therapeutics

A number of constructs have been devised to date for the production of recombinant TCRs. These constructs fall into two broad classes, single-chain TCRs and dimeric TCRs, the literature relevant to these constructs is summarised below.

Single-chain TCRs (scTCRs) are artificial constructs consisting of a single amino acid strand, which like native heterodimeric TCRs bind to MHC-peptide complexes. Unfortunately, attempts to produce functional alpha/beta analogue scTCRs by simply linking the alpha and beta chains such that both are expressed in a single open reading frame have been unsuccessful, presumably because of the natural instability of the alpha-beta soluble domain pairing.

Accordingly, special techniques using various truncations of either or both of the alpha and beta chains have been necessary for the production of scTCRs. These formats appear to be applicable only to a very limited range of scTCR sequences. Soo Hoo *et*

al (1992) PNAS. **89** (10): 4759-63 report the expression of a mouse TCR in single chain format from the 2C T cell clone using a truncated beta and alpha chain linked with a 25 amino acid linker and bacterial periplasmic expression (see also Schodin *et al* (1996) Mol. Immunol. **33 (9)**: 819-29). This design also forms the basis of the m6 single-chain TCR reported by Holler *et al* (2000) PNAS. **97** (10): 5387-92 which is derived from the 2C scTCR and binds to the same H2-Ld-restricted alloepitope. Shusta *et al* (2000) Nature Biotechnology **18**: 754-759 and US 6,423,538 report using a murine single-chain 2C TCR constructs in yeast display experiments, which produced mutated TCRs with, enhanced thermal stability and solubility. This report also demonstrated the ability of these displayed 2C TCRs to selectively bind cells expressing their cognate pMHC. Khandekar *et al* (1997) J. Biol. Chem. **272** (51): 32190-7 report a similar design for the murine D10 TCR, although this scTCR was fused to MBP and expressed in bacterial cytoplasm (see also Hare *et al* (1999) Nat. Struct. Biol. **6** (6): 574-81). Hilyard *et al* (1994) PNAS. **91** (19): 9057-61 report a human scTCR specific for influenza matrix protein-HLA-A2, using a Vα-linker-Vβ design and expressed in bacterial periplasm.

Chung *et al* (1994) PNAS. **91** (26) 12654-8 report the production of a human scTCR using a Vα-linker-Vβ-Cβ design and expression on the surface of a mammalian cell line. This report does not include any reference to peptide-HLA specific binding of the scTCR. Plaksin *et al* (1997) J. Immunol. **158** (5): 2218-27 report a similar Vα-linker-Vβ-Cβ design for producing a murine scTCR specific for an HIV gp120-H-2D^{d} epitope. This scTCR is expressed as bacterial inclusion bodies and refolded *in vitro*.

A number of papers describe the production of TCR heterodimers which include the native disulphide bridge which connects the respective subunits (Garboczi, *et al.*, (1996), Nature **384**(6605): 134-41; Garboczi, *et al.*, (1996), J Immunol **157**(12): 5403-10; Chang *et al.*, (1994), PNAS USA **91**: 11408-11412; Davodeau *et al.*, (1993), *J. Biol. Chem.* **268**(21): 15455-15460; Golden *et al.*, (1997), J. Imm. Meth. **206**: 163-169; US Patent No. 6080840). However, although such TCRs can be recognised by

TCR-specific antibodies, none were shown to recognise its native ligand at anything other than relatively high concentrations and/or were not stable.

In WO 99/60120, a soluble TCR is described which is correctly folded so that it is capable of recognising its native ligand, is stable over a period of time, and can be produced in reasonable quantities. This TCR comprises a TCR α or γ chain extracellular domain dimerised to a TCR β or δ chain extracellular domain respectively, by means of a pair of C-terminal dimerisation peptides, such as leucine zippers. This strategy of producing TCRs is generally applicable to all TCRs.

Reiter *et al*, Immunity, 1995, 2:281-287, details the construction of a soluble molecule comprising disulphide-stabilised TCR α and β variable domains, one of which is linked to a truncated form of Pseudomonas exotoxin (PE38). One of the stated reasons for producing this molecule was to overcome the inherent instability of single-chain TCRs. The position of the novel disulphide bond in the TCR variable domains was identified via homology with the variable domains of antibodies, into which these have previously been introduced (for example see Brinkmann, *et al.* (1993), Proc. Natl. Acad. Sci. USA **90:** 7538-7542, and Reiter, *et al.* (1994) Biochemistry **33:** 5451-5459). However, as there is no such homology between antibody and TCR constant domains, such a technique could not be employed to identify appropriate sites for new inter-chain disulphide bonds between TCR constant domains.

As mentioned above Shusta *et al* (2000) Nature Biotechnology **18**: 754-759 report using single-chain 2 C TCR constructs in yeast display experiments. The principle of displaying scTCRs on phage particles has previously been discussed. For example, WO 99/19129 details the production of scTCRs, and summarise a potential method for the production of phage particles displaying scTCRs of the Vα-Linker-Vβ Cβ format. However, this application contains no exemplification demonstrating the production of said phage particles displaying TCR. The application does however refer to a co-pending application:

"The construction of DNA vectors including a DNA segment encoding a sc-TCR molecules fused to a bacteriophage coat protein (gene II or gene VIII) have been described in said pending U.S. application No. 08/813,781."

Furthermore, this application relies on the ability of anti-TCR antibodies or super-antigen MHC complexes to recognise the soluble, non-phage displayed, scTCRs produced to verify their correct conformation. Therefore, true peptide-MHC binding specificity of the scTCRs, in any format, is not conclusively demonstrated.

Screening Use

A number of important cellular interactions and cell responses, including the TCR-mediated immune synapse, are controlled by contacts made between cell surface receptors and ligands presented on the surfaces of other cells. These types of specific molecular contacts are of crucial importance to the correct biochemical regulation in the human body and are therefore being studied intensely. In many cases, the objective of such studies is to devise a means of modulating cellular responses in order to prevent or combat disease.

Therefore, methods with which to identify compounds that bind with some degree of specificity to human receptor or ligand molecules are important as leads for the discovery and development of new disease therapeutics. In particular, compounds that interfere with certain receptor-ligand interactions have immediate potential as therapeutic agents or carriers.

Advances in combinatorial chemistry, enabling relatively easy and cost-efficient production of very large compound libraries have increased the scope for compound testing enormously. Now the limitations of screening programmes most often reside in the nature of the assays that can be employed, the production of suitable receptor and ligand molecules and how well these assays can be adapted to high throughput screening methods.

Display Methods

It is often desirable to present a given peptide or polypeptide on the surface of a proteinaceous particle. Such particles may serve as purification aids for the peptide or polypeptide (since the particles carrying the peptide or polypeptide may be separated from unwanted contaminants by sedimentation or other methods). They may also serve as particulate vaccines, the immune response to the surface displayed peptide or polypeptide being stimulated by the particulate presentation. Protein p24 of the yeast retrotransposon, and the hepatitis B surface coat protein are examples of proteins which self assemble into particles. Fusion of the peptide or polypeptide of interest to these particle-forming proteins is a recognised way of presenting the peptide or polypeptide on the surface of the resultant particles.

However, particle display methods have primarily been used to identify proteins with desirable properties such as enhanced expression yields, binding and/or stability characteristics. These methods involve creating a diverse pool or 'library' of proteins or polypeptides expressed on the surface of proteinaceous particles. These particles have two key features, firstly each particle presents a single variant protein or polypeptide, and secondly the genetic material encoding the expressed protein or polypeptide is associated with that of the particle. This library is then subjected to one or more rounds of selection. For example, this may consist of contacting a ligand with a particle-display library of mutated receptors and identifying which mutated receptors bind the ligand with the highest affinity. Once the selection process has been completed the receptor or receptors with the desired properties can be isolated, and their genetic material can be amplified in order to allow the receptors to be sequenced.

These display methods fall into two broad categories, *in-vitro* and *in-vivo* display.

All *in-vivo* display methods rely on a step in which the library, usually encoded in or with the genetic nucleic acid of a replicable particle such as a plasmid or phage replicon is transformed into cells to allow expression of the proteins or polypeptides. (Plückthun (2001) Adv Protein Chem **55** 367-403). There are a number of

replicon/host systems that have proved suitable for *in-vivo* display of protein or polypeptides. These include the following

Phage / bacterial cells

plasmid / CHO cells

Vectors based on the yeast 2μm plasmid / yeast cells

bacculovirus / insect cells

plasmid / bacterial cells

In-vivo display methods include cell-surface display methods in which a plasmid is introduced into the host cell encoding a fusion protein consisting of the protein or polypeptide of interest fused to a cell surface protein or polypeptide. The expression of this fusion protein leads to the protein or polypeptide of interest being displayed on the surface of the cell. The cells displaying these proteins or polypeptides of interest can then be subjected to a selection process such as FACS and the plasmids obtained from the selected cell or cells can be isolated and sequenced. Cell surface display systems have been devised for mammalian cells (Higuschi (1997) J Immunol. Methods **202** 193-204), yeast cells (Shusta (1999) J Mol Biol **292** 949-956) and bacterial cells (Sameulson (2002) J. Biotechnol **96** (2) 129-154).

Numerous reviews of the various in-vivo display techniques have been published. For example, (Hudson (2002) Expert Opin Biol Ther (2001) **1** (5) 845-55) and (Schmitz (2000) **21** (Supp A) S106-S112).

In-vitro display methods are based on the use of ribosomes to translate libraries of mRNA into a diverse array of protein or polypeptide variants. The linkage between the proteins or polypeptides formed and the mRNA encoding these molecules is maintained by one of two methods. Conventional ribosome display utilises mRNA sequences that encode a short (typically 40-100 amino acid) linker sequence and the protein or polypeptide to be displayed. The linker sequence allow the displayed

protein or polypeptide sufficient space to re-fold without being sterically hindered by the ribosme. The mRNA sequence lacks a 'stop' codon, this ensures that the expressed protein or polypeptide and the RNA remain attached to the ribosome particle. The related mRNA display method is based on the preparation of mRNA sequences encoding the protein or polypeptide of interest and DNA linkers carrying a puromycin moiety. As soon as the ribosome reaches the mRNA/DNA junction translation is stalled and the puromycin forms a covalent linkage to the ribosome. For a recent review of these two related *in-vitro* display methods see (Amstutz (2001) Curr Opin Biotechnol **12** 400-405).

Particularly preferred is the phage display technique which is based on the ability of bacteriophage particles to express a heterologous peptide or polypeptide fused to their surface proteins. (Smith (1985) Science **217** 1315-1317). The procedure is quite general, and well understood in the art for the display of polypeptide monomers. However, in the case of polypeptides that in their native form associate as dimers, only the phage display of antibodies appears to have been thoroughly investigated.

For monomeric polypeptide display there are two main procedures:
Firstly (Method A) by inserting into a vector (phagemid) DNA encoding the heterologous peptide or polypeptide fused to the DNA encoding a bacteriophage coat protein. The expression of phage particles displaying the heterologous peptide or polypeptide is then carried out by transfecting bacterial cells with the phagemid, and then infecting the transformed cells with a 'helper phage'. The helper phage acts as a source of the phage proteins not encoded by the phagemid required to produce a functional phage particle.

Secondly (Method B), by inserting DNA encoding the heterologous peptide or polypeptide into a complete phage genome fused to the DNA encoding a bacteriophage coat protein. The expression of phage particles displaying the heterologous peptide or polypeptide is then carried out by infecting bacterial cells with the phage genome. This method has the advantage of the first method of being a

'single-step' process. However, the size of the heterologous DNA sequence that can be successfully packaged into the resuting phage particles is reduced. M13, T7 and Lambda are examples of suitable phages for this method.

A variation on (Method B) the involves adding a DNA sequence encoding a nucleotide binding domain to the DNA in the phage genome encoding the heterologous peptide be displayed, and further adding the corresponding nucleotide binding site to the phage genome. This causes the heterologous peptide to become directly attached to the phage genome. This peptide/genome complex is then packaged into a phage particle which displays the heterologous peptide. This method is fully described in WO 99/11785.

The phage particles can then be recovered and used to study the binding characteristics of the heterologous peptide or polypeptide. Once isolated, phagemid or phage DNA can be recovered from the peptide- or polypeptide-displaying phage particle, and this DNA can be replicated via PCR. The PCR product can be used to sequence the heterologous peptide or polypeptide displayed by a given phage particle.

The phage display of single-chain antibodies and fragments thereof, has become a routine means of studying the binding characteristics of these polypeptides. There are numerous books available that review phage display techniques and the biology of the bacteriophage. (See, for example, Phage Display – A Laboratory Manual, Barbas *et al.*, (2001) Cold Spring Harbour Laboratory Press).

A third phage display method (Method C) relies on the fact that heterologous polypeptides having a cysteine residue at a desired location can be expressed in a soluble form by a phagemid or phage genome, and caused to associate with a modified phage surface protein also having a cysteine residue at a surface exposed position, via the formation of a disulphide linkage between the two cysteines. WO 01/ 05950 details the use of this alternative linkage method for the expression of single-chain antibody-derived peptides.

Brief Description of the Invention

Native TCR's are heterodimers which have lengthy transmembrane domains which are essential to maintain their stability as functional dimers. As discussed above, TCRs are useful for research and therapeutic purposes in their soluble forms so display of the insoluble native form has little utility. On the other hand, soluble stable forms of TCRs have proved difficult to design, and since most display methods appear to have been described only for monomeric peptides and polypeptides, display methods suitable for soluble dimeric TCRs have not been investigated. Furthermore, since the functionality of the displayed TCR depends on proper association of the variable regions of the TCR dimer, successful display of a functional dimeric TCR is not trivial.

WO 99/18129 contains the statement : "DNA constructs encoding the sc-TCR fusion proteins can be used to make a bacteriophage display library in accordance with methods described in pending U.S. application Serial No. 08/813.781 filed on March 7, 1997, the disclosure of which is incorporated herein by reference.", but no actual description of such display is included in this application. However, The inventors of this application published a paper (Weidanz (1998) J Immunol Methods 221 59-76) that demonstrates the display of two murine scTCRs on phage particles.

WO 01/62908 discloses methods for the phage display of scTCRs and scTCR/ Ig fusion proteins. However, the functionality (specific pMHC binding) of the constructs disclosed was not assessed.

This invention is based in part on the finding that single chain and dimeric TCRs can be expressed as surface fusions to proteinaceous particles, and makes available proteinaceous particles displaying alpha/beta-analogue and gamma/delta-analogue scTCR and dTCR constructs. The proteinaceous particles on which the TCRs are displayed include self-aggregating particle-forming proteins, phage, virus-derived and ribosome particles. Such proteinaceous particle-displayed TCRs are useful for purification and screening purposes, particularly as a diverse library of particle

displayed TCRs for biopanning to identify TCRs with desirable characteristics such as strong affinity for the target MHC-peptide complex. In the latter connection, particle-displayed scTCRs may be useful for identification of the desired TCR, but that information may be better applied to the construction of analogous dimeric TCRs for ultimate use in therapy.

Detailed Description of the Invention

The present invention provides a proteinaceous particle displaying on its surface a T-cell receptor(TCR), characterised in that

(i) the proteinaceous particle is a ribosome and the TCR is a single chain TCR (scTCR) polypeptide, or

(ii) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a human scTCR or a human dimeric T-cell receptor (dTCR) polypeptide pair, or

(iii) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a non-human dTCR polypeptide pair, or

(iv) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a scTCR polypeptide comprising TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain, and a disulfide bond which has no equivalent in native T cell receptors links residues of the constant region sequences.

In a preferred embodiment, the invention provides a proteinaceous particle displaying on its surface a dimeric T-cell receptor (dTCR) polypeptide pair, or a single chain T-cell receptor (scTCR) polypeptide wherein

the dTCR polypeptide pair is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains, and the scTCR is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain;

the variable region sequences of the dTCR polypeptide pair or scTCR polypeptide are mutually orientated substantially as in native TCRs; and

in the case of the scTCR polypeptide a disulfide bond which has no equivalent in native T cell receptors links residues of the polypeptide.

In the case of αβ scTCRs or dTCRs displayed according to the invention, the requirement that the variable region sequences of the α and β segments are mutually orientated substantially as in native αβ T cell receptors is tested by confirming that the molecule binds to the relevant TCR ligand (pMHC complex, CD1-antigen complex, superantigen or superantigen/pMHC complex) - if it binds, then the requirement is met. Interactions with pMHC complexes can be measured using a BIAcore 3000™ or BIAcore 2000™ instrument. WO99/6120 provides detailed descriptions of the methods required to analyse TCR binding to MHC-peptide complexes. These methods are equally applicable to the study of TCR/ CD1 and TCR/superantigen interactions. In order to apply these methods to the study of TCR/CD1 interactions soluble forms of CD1 are required, the production of which are described in (Bauer (1997) Eur J Immunol 27 (6) 1366-1373). In the case of γδ TCRs of the present

invention the cognate ligands for these molecules are unknown therefore secondary means of verifying their conformation such as recognition by antibodies can be employed. The monoclonal antibody MCA991T (available from Serotec), specific for the δ chain variable region, is an example of an antibody appropriate for this task.

The scTCRs or dTCRs of the present invention may be displayed on phage particles by, for example, the following two means:

(i) The C=terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, can be directly linked by a peptide bond to a surface exposed residue of the proteinaceous particle. For example, the said surface exposed residue is preferably at the N-terminus of the gene product of bacteriophage gene III or gene VIII; and

(ii) The C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, is linked by a disulfide bond to a surface exposed cysteine residue of the proteinaceous particle via an introduced cysteine residue. For example, the said surface exposed residue is again preferably at the N-terminus of the gene product of bacteriophage gene III or gene VIII.

Method (i) above is preferred. In the case of a scTCR, nucleic acid encoding the TCR may be fused to nucleic acid encoding the particle forming protein or a surface protein of the replicable particle such as a phage. Alternatively, nucleic acid representing mRNA but without a stop codon, or fused to puromycin RNA may be translated by ribosome such that the TCR remains fused to the ribosome particle. In the case of a dTCR, nucleic acid encoding one chain of the TCR may be fused tonucleic acid encoding the particle forming protein or a surface protein of the replicable particle such as a phage, and the second chain of the TCR polypeptide pair may be allowed to associate with the resultant expressed particle displaying the first chain. Proper functional association of the two chains is assisted by the presence of cysteines in the

constant region of the two chains which are capable of forming an interchain disulfide bond, as more fully discussed below.

The displayed scTCR

The displayed scTCR polypeptide may be, for example, one which has

a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant region extracellular sequence,

a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region fused to the N terminus of an amino acid sequence corresponding to TCR β chain constant region extracellular sequence,

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment, or vice versa, and

a disulfide bond between the first and second chains, said disulfide bond being one which has no equivalent in native αβ or γδ T cell receptors,

the length of the linker sequence and the position of the disulfide bond being such that the variable region sequences of the first and second segments are mutually orientated substantially as in native αβ or γδ T cell receptors.

The displayed dTCR

The dTCR which is displayed on the proteinaceous particle may be one which is constituted by

a first polypeptide wherein a sequence corresponding to a TCR α or δ chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant region extracellular sequence, and

a second polypeptide wherein a sequence corresponding to a TCR β or γ chain variable region sequence fused to the N terminus a sequence corresponding to a TCR β chain constant region extracellular sequence,

the first and second polypeptides being linked by a disulfide bond which has no equivalent in native αβ or γδ T cell receptors.

dTCR Polypeptide Pair and scTCR Polypeptide

The constant region extracellular sequences present in the scTCRs or dTCRs preferably correspond to those of a human TCR, as do the variable region sequences. However, the correspondence between such sequences need not be 1:1 on an amino acid level. N- or C-truncation, and/or amino acid deletion and/or substitution relative to the corresponding human TCR sequences is acceptable, provided the overall result is mutual orientation of the α and β variable region sequences, or γ and δ variable region sequences is as in native αβ, or γδ T cell receptors respectively. In particular, because the constant region extracellular sequences present in the first and second segments are not directly involved in contacts with the ligand to which the scTCR or dTCR binds, they may be shorter than, or may contain substitutions or deletions relative to, extracellular constant domain sequences of native TCRs.

The constant region extracellular sequence present in one of the dTCR polypeptide pair, or in the first segment of a scTCR polypeptide may include a sequence corresponding to the extracellular constant Ig domain of a TCR α chain, and/or the constant region extracellular sequence present in the other member of the pair or second segment may include a sequence corresponding to the extracellular constant Ig domain of a TCR β chain.

In one embodiment of the invention, one member of the polypeptide pair or the first segment of the scTCR polypeptide corresponds to substantially all the variable region

of a TCR α chain fused to the N terminus of substantially all the extracellular domain of the constant region of an TCR α chain; and/or the other member of the pair or second segment corresponds to substantially all the variable region of a TCR β chain fused to the N terminus of substantially all the extracellular domain of the constant region of a TCR β chain.

In another embodiment, the constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide correspond to the constant regions of the α and β chains of a native TCR truncated at their C termini such that the cysteine residues which form the native inter-chain disulfide bond of the TCR are excluded. Alternatively those cysteine residues may be substituted by another amino acid residue such as serine or alanine, so that the native disulfide bond is deleted. In addition, the native TCR β chain contains an unpaired cysteine residue and that residue may be deleted from, or replaced by a non-cysteine residue in, the β sequence of the scTCR of the invention.

In one particular embodiment of the invention, the TCR α and β chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may together correspond to the functional variable domain of a first TCR, and the TCR α and β chain constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a second TCR, the first and second TCRs being from the same species. Thus the α and β chain variable region sequences present in dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a first human TCR, and the α and β chain constant region extracellular sequences may correspond to those of a second human TCR. For example, A6 Tax sTCR constant region extracellular sequences can be used as a framework onto which heterologous α and β variable domains can be fused.

In another embodiment of the invention, the TCR δ and γ chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide respectively, may together correspond to the functional variable domain of a first TCR, and the TCR α and β chain constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide respectively, may correspond to those of a second TCR, the first and second TCRs being from the same species. Thus the δ and γ chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a first human TCR, and the α and β chain constant region extracellular sequences may correspond to those of a second human TCR. For example, A6 Tax sTCR constant region extracellular sequences can be used as a framework onto which heterologous γ and δ variable domains can be fused.

In one particular embodiment of the invention, the TCR α and β, or δ and γ chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may together correspond to the functional variable domain of a first human TCR, and the TCR α and β chain constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a second non-human TCR, Thus the α and β, or δ and γ chain variable region sequences present dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a first human TCR, and the α and β chain constant region extracellular sequences may correspond to those of a second non-human TCR. For example, murine TCR constant region extracellular sequences can be used as a framework onto which heterologous human α and β TCR variable domains can be fused.

Linker in the scTCR Polypeptide

For scTCR-displaying proteinaceous particles of the present invention, a linker sequence links the first and second TCR segments, to form a single polypeptide strand.

The linker sequence may, for example, have the formula -P-AA-P- wherein P is proline and AA represents an amino acid sequence wherein the amino acids are glycine and serine.

For the scTCR displayed by proteinaceous particles of the present invention to bind to a ligand, MHC-peptide complex in the case of αβ TCRs, the first and second segments must be paired so that the variable region sequences thereof are orientated for such binding. Hence the linker should have sufficient length to span the distance between the C terminus of the first segment and the N terminus of the second segment, or vice versa. On the other hand excessive linker length should preferably be avoided, in case the end of the linker at the N-terminal variable region sequence blocks or reduces bonding of the scTCR to the target ligand.

For example, in the case where the constant region extracellular sequences present in the first and second segments correspond to the constant regions of the α and β chains of a native TCR truncated at their C termini such that the cysteine residues which form the native interchain disulfide bond of the TCR are excluded, and the linker sequence links the C terminus of the first segment to the N terminus of the second segment, the linker may consist of from 26 to 41, for example 29, 30, 31 or 32 amino acids, and a particular linker has the formula -PGGG-$(SGGGG)_5$-P- wherein P is proline, G is glycine and S is serine.

Inter-chain Disulfide bond

A principle characterising feature of the scTCRs displayed by proteinaceous particles of the present invention, and preferably a feature of the displayed dTCRs, is ~~the~~ a disulfide bond between the constant region extracellular sequences of the dTCR polypeptide pair or first and second segments of the scTCR polypeptide. That bond may correspond to the native inter-chain disulfide bond present in native dimeric αβ TCRs, or may have no counterpart in native TCRs, being between cysteines specifically incorporated into the constant region extracellular sequences of dTCR

polypeptide pair or first and second segments of the scTCR polypeptide. In some cases, both a native and a non-native disulfide bond may be desirable.

The position of the disulfide bond is subject to the requirement that the variable region sequences of dTCR polypeptide pair or first and second segments of the scTCR polypeptide are mutually orientated substantially as in native αβ or γδ T cell receptors.

The disulfide bond may be formed by mutating non-cysteine residues on the first and second segments to cysteine, and causing the bond to be formed between the mutated residues. Residues whose respective β carbons are approximately 6 Å (0.6 nm) or less, and preferably in the range 3.5 Å (0.35 nm) to 5.9 Å (0.59 nm) apart in the native TCR are preferred, such that a disulfide bond can be formed between cysteine residues introduced in place of the native residues. It is preferred if the disulfide bond is between residues in the constant immunoglobulin region, although it could be between residues of the membrane proximal region. Preferred sites where cysteines can be introduced to form the disulfide bond are the following residues in exon 1 of TRAC*01 for the TCR α chain and TRBC1*01 or TRBC2*01 for the TCR β chain:

TCR α chain	TCR β chain	Native β carbon separation (nm)
Thr 48	Ser 57	0.473
Thr 45	Ser 77	0.533
Tyr 10	Ser 17	0.359
Thr 45	Asp 59	0.560
Ser 15	Glu 15	0.59

Now that the residues in human TCRs which can be mutated into cysteine residues to form a new interchain disulfide bond in dTCRs or scTCRs displayed according to the invention have been identified, those of skill in the art will be able to mutate TCRs of other species in the same way to produce a dTCR or scTCR of that species for phage display. In humans, the skilled person merely needs to look for the following motifs

in the respective TCR chains to identify the residue to be mutated (the shaded residue is the residue for mutation to a cysteine).

α Chain Thr 48: DSDVYITDKTVLDMRSMDFK (amino acids 39-58 of exon 1 of the TRAC*01 gene)

α Chain Thr 45: QSKDSDVYITDKTVLDMRSM(amino acids 36-55 of exon 1 of the TRAC*01 gene)

α Chain Tyr 10: DIQNPDPAVYQLRDSKSSDK(amino acids 1-20 of exon 1 of the TRAC*01 gene)

α Chain Ser 15: DPAVYQLRDSKSSDKSVCLF(amino acids 6-25 of exon 1 of the TRAC*01 gene)

β Chain Ser 57: NGKEVHSGVSTDPQPLKEQP(amino acids 48- 67 of exon 1 of the TRBC1*01 & TRBC2*01 genes)

β Chain Ser 77: ALNDSRYALSSRLRVSATFW(amino acids 68- 87 of exon 1 of the TRBC1*01 & TRBC2*01 genes)

β Chain Ser 17: PPEVAVFEPSEAEISHTQKA(amino acids 8- 27 of exon 1 of the TRBC1*01 & TRBC2*01 genes)

β Chain Asp 59: KEVHSGVSTDPQPLKEQPAL(amino acids 50- 69 of exon 1 of the TRBC1*01 & TRBC2*01 genes gene)

β Chain Glu 15: VFPPEVAVFEPSEAEISHTQ(amino acids 6- 25 of exon 1 of the TRBC1*01 & TRBC2*01 genes)

In other species, the TCR chains may not have a region which has 100% identity to the above motifs. However, those of skill in the art will be able to use the above motifs to identify the equivalent part of the TCR α or β chain and hence the residue to be mutated to cysteine. Alignment techniques may be used in this respect. For example, ClustalW, available on the European Bioinformatics Institute website (http://www.ebi.ac.uk/index.html) can be used to compare the motifs above to a particular TCR chain sequence in order to locate the relevant part of the TCR sequence for mutation.

The present invention includes within its scope proteinaceous particle-displayed αβ and γδ-analogue scTCRs, as well as those of other mammals, including, but not limited to, mouse, rat, pig, goat and sheep. As mentioned above, those of skill in the art will be able to determine sites equivalent to the above-described human sites at which cysteine residues can be introduced to form an inter-chain disulfide bond. For example, the following shows the amino acid sequences of the mouse Cα and Cβ soluble domains, together with motifs showing the murine residues equivalent to the human residues mentioned above that can be mutated to cysteines to form a TCR interchain disulfide bond (where the relevant residues are shaded):

Mouse Cα soluble domain:

PYIQNPEPAVYQLKDPRSQDSTLCLFTDFDSQINVPKTMESGTFITDKTVLDMK
AMDSKSNGAIAWSNQTSFTCQDIFKETNATYPSSDVP

Mouse Cβ soluble domain:

EDLRNVTPPKVSLFEPSKAEIANKQKATLVCLARGFFPDHVELSWWVNGREV
HSGVSTDPQAYKESNYSYCLSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDK
WPEGSPKPVTQNISAEAWGRAD

Murine equivalent of human α Chain Thr 48: ESGTFITDKTVLDMKAMDSK

Murine equivalent of human α Chain Thr 45: KTMESGTFITDKTVLDMKAM

Murine equivalent of human α Chain Tyr 10: YIQNPEPAVYQLKDPRSQDS

Murine equivalent of human α Chain Ser 15: AVYQLKDPRSQDSTLCLFTD

Murine equivalent of human β Chain Ser 57: NGREVHSGVSTDPQAYKESN

Murine equivalent of human β Chain Ser 77: KESNYSYCLSSRLRVSATFW

Murine equivalent of human β Chain Ser 17: PPKVSLFEPSKAEIANKQKA

Murine equivalent of human β Chain Asp 59: REVHSGVSTDPQAYKESNYS

Murine equivalent of human β Chain Glu 15: VTPPKVSLFEPSKAEIANKQ

As discussed above, the A6 Tax sTCR extracellular constant regions can be used as framework onto which heterologous variable domains can be fused. It is preferred that the heterologous variable region sequences are linked to the constant region sequences at any point between the disulfide bond and the N termini of the constant region sequences. In the case of the A6 Tax TCR α and β constant region sequences, the disulfide bond may be formed between cysteine residues introduced at amino acid residues 158 and 172 respectively. Therefore it is preferred if the heterologous α and β chain variable region sequence attachment points are between residues 159 or 173 and the N terminus of the α or β constant region sequences respectively.

TCR Display.

The preferred *in-vivo* TCR display method for biopanning to identify TCRs having desirable properties such as strong affinity for a target peptide-MHC complex is phage display.

Firstly, a DNA library is constructed that encodes a diverse array of mutated TCRs. This library is constructed by using DNA encoding a native TCR as the template for amplification. There are a number of suitable methods, known to those skilled in the art, for the introduction of the desired mutations into the TCR DNA, and hence the finally expressed TCR protein.. For example error-prone PCR (EP-TCR), DNA shuffling techniques, and the use of bacterial mutator strains such as XL-1-Red are convenient means of introducing mutations into the TCR sequences. It is particularly preferred-if these mutations are introduced into defined regions of the TCRs. For example, mutations in the complementarity-determining regions (CDRs) are likely to be the most appropriate for the production of TCRs with enhanced ligand-binding properties. EP-PCR is an example of a method by which such 'region-specific' mutations can be introduced into the TCRs. EP-PCR primers are used that are complementary to DNA sequences bordering the region to be mutated to amplify multiple copies of this region of the TCR DNA that contain a controllable level of random mutations. These DNA sequences encoding mutated regions are inserted into the DNA sequences, which encode the non-mutagenised sections of the TCR, by ligation or overlapping PCR. The DNA encoding the TCR with mutated region can then be ligated onto DNA encoding a heterologous polypeptide in order to produce a fusion protein suitable for display. For example, the TCR DNA can be ligated onto DNA encoding the GIII or GVIII surface protein for use in phage display applications. In the case of a scTCR such ligation is performed as for phage display of any monomeric peptide or polypeptide. In the case of dTCRs, only one of the TCR chains is ligated as aforesaid. The other chain is encoded in nucleic acid for co-expression with the phagemid and helper phage nucleic acid, so that the expressed second chain finds and associates with the expressed phage with surface displayed first chain. In both cases, as discussed in more detail above, properly positioned cysteines in the constant regions are helpful in causing the variable regions of the TCR to adopt their functional positions, through the formation of a disulfide bond by those cysteines.

For expression, the plasmids comprising the DNA library are then contacted with cells capable of causing the expression of the encoded genetic material under conditions

suitable to allow the transformation of said cells. The transformed cells are then incubated to allow the expression of the TCR-displaying proteinaceous particles. These particles can then be used for screening or in assays to identify TCR variants with specific enhanced characteristics. Any particles that possess the enhanced characteristics under investigation can then be isolated. The DNA encoding these TCRs can then be amplified by PCR and the sequence determined.

As mentioned above, for scTCR phage display, the scTCR polypeptide is expressed according to any of the three general prior art techniques discussed earlier as Methods A, B, and C. For the scTCRs displayed by phage particles of the present invention to bind to a ligand, MHC-peptide complex in the case of αβ TCRs, the first and second segments must be paired so that the variable region sequences thereof are orientated for such binding. This correct pairing is assisted by the introduction of a disulfide bond in the extracellular constant region of the scTCR. Without wanting to be limited by theory, the novel disulfide bond is believed to provide extra stability to the scTCR during the folding process and thereby facilitating correct pairing of the first and second segments.

Also as mentioned above, for dTCR phage display, one of the dTCR polypeptide pair is expressed according to any of the three general prior art techniques discussed earlier as Methods A, B, and C as if it were eventually to be displayed as a monomeric polypeptide on the phage, and the other of the dTCR polypeptide pair is co-expressed in the same host cell. As the phage particle self assembles, the two polypeptides self associate for display as a dimer on the phage. Again, in the preferred embodiment of this aspect of the invention, correct folding during association of the polypeptide pair is assisted by a disulfide bond between the constant sequences, as discussed above. Further details of a procedure for phage display of a dTCR having an interchain disulfide bond appear in the Examples herein.

As an alternative, the phage displaying the first chain of the TCR may be expressed first, and the second chain polypeptide may be contacted with the expressed phage in a subsequent step, for association as a functional TCR on the phage surface.

The preferred *in-vitro* TCR display method for biopanning to identify TCRs having desirable properties such as strong affinity for a target peptide-MHC complex is ribosomal display of scTCRs.

Firstly, a DNA library is constructed that encodes a diverse array of mutated TCRs using the techniques discussed above .

The DNA library is then contacted with RNA polymerase in order to produce a complementary mRNA library. Optionally, for mRNA display techniques the mRNA sequences can then be ligated to a DNA sequence comprising a puromycin binding site. These genetic constructs are then contacted with ribosomes in-vitro under conditions allowing the translation of the scTCR polypeptide. These scTCR-displaying ribosomes can then used for screening or in assays to identify TCR variants with specific enhanced characteristics. Any particles that possess the enhanced characteristics under investigation can then be isolated. The mRNA encoding these TCRs can then converted to the complementary DNA sequences using reverse transcriptase. This DNA can then be amplified by PCR and the sequence determined.

Additional Aspects

A proteinaceous particle displaying a scTCR or dTCR (which preferably is constituted by constant and variable sequences corresponding to human sequences) of the present invention may be provided in substantially pure form, or as a purified or isolated preparation. For example, it may be provided in a form which is substantially free of other proteins.

A phage particle displaying a plurality of scTCRs or dTCRs of the present invention may be provided in a multivalent complex. Thus, the present invention provides, in

one aspect, a multivalent T cell receptor (TCR) complex, which comprises a phage particle displaying a plurality of scTCRs or dTCRs as described herein. Each of the plurality of said scTCRs or dTCRs is preferably identical.

In a further aspect, the invention provides a method for detecting TCR ligand complexes, which comprises:

a. providing a TCR-displaying pproteineaceous particle of the current invention
b. contacting the TCR-displaying phage with the putative ligand complexes; and
detecting binding of the TCR-displaying proteinaceous particle to the putative ligand complexes.

TCR ligands suitable for identification by the above method include, but are not limited to, peptide-MHC complexes.

Isolation of TCR variants with enhanced characteristics

A further aspect of the invention is a method for the isolation of TCRs with a specific enhanced characteristic, said method comprising subjecting a diverse library of TCRs displayed on proteinaceous particles to an assay which measures said characteristic and thereby identifying those proteinaceous particles which display a TCR with the desired enhancement and isolating these proteinaceous particles. The DNA sequences encoding the variant TCRs can then be obtained and amplified by PCR to allow the sequences to be determined. The characteristics that can be enhanced include, but are not limited to, ligand binding affinity and construct stability.

Screening Use

The TCR-displaying proteinaceous particles of the present invention are capable of utilisation in screening methods designed to identify modulators, including inhibitors, of the TCR-mediated cellular immune synapse.

As is know to those skilled in the art there are a number of assay formats that provide a suitable basis for protein-protein interaction screens of this type.

Amplified Luminescent Proximity Homogeneous Assay systems such as the AlphaScreen™, rely on the use of "Donor" and "Acceptor" beads that are coated with a layer of hydrogel to which receptor and ligand proteins can be attached. The interaction between these receptor and ligand molecules brings the beads into proximity. When these beads are subject to laser light a photosensitizer in the "Donor" bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a chemiluminescer in the "Acceptor" bead that further activates fluorophores contained within the same bead. The fluorophores subsequently emit light at 520-620 nm, this signals that the receptor-ligand interaction has occurred. The presence of an inhibitor of the receptor-ligand interaction causes this signal to be diminished.

Surface Plasmon Resonance (SPR) is an interfacial optical assay, in which one binding partner (normally the receptor) is immobilised on a 'chip' (the sensor surface) and the binding of the other binding partner (normally the ligand), which is soluble and is caused to flow over the chip, is detected. The binding of the ligand results in an increase in concentration of protein near to the chip surface which causes a change in the refractive index in that region. The surface of the chip is comprised such that the change in refractive index may be detected by surface plasmon resonance, an optical phenomenon whereby light at a certain angle of incidence on a thin metal film produces a reflected beam of reduced intensity due to the resonant excitation of waves of oscillating surface charge density (surface plasmons). The resonance is very sensitive to changes in the refractive index on the far side of the metal film, and it is this signal which is used to detect binding between the immobilised and soluble proteins. Systems which allow convenient use of SPR detection of molecular interactions, and data analysis, are commercially available. Examples are the Iasys™ machines (Fisons) and the Biacore™ machines.

Other interfacial optical assays include total internal reflectance fluorescence (TIRF), resonant mirror (RM) and optical grating coupler sensor (GCS), and are discussed in more detail in Woodbury and Venton (*J. Chromatog. B.* **725** 113-137 (1999)).

The scintillation proximity assay (SPA) has been used to screen compound libraries for inhibitors of the low affinity interaction between CD28 and B7 (K_d probably in the region of 4 μM (Van der Merwe *et al* J. Exp. Med. **185**:393-403 (1997), Jenh *et al*, Anal Biochem **165**(2) 287-93 (1998)). SPA is a radioactive assay making use of beta particle emission from certain radioactive isotopes which transfers energy to a scintillant immobilised on the indicator surface. The short range of the beta particles in solution ensures that scintillation only occurs when the beta particles are emitted in close proximity to the scintillant. When applied for the detection of protein-protein interactions, one interaction partner is labelled with the radioisotope, while the other is either bound to beads containing scintillant or coated on a surface together with scintillant. If the assay can be set up optimally, the radioisotope will be brought close enough to the scintillant for photon emission to be activated only when binding between the two proteins occurs.

A further aspect of the invention is a method of identifying an inhibitor of the interaction between a TCR-displaying proteinaceous particle of the invention, and a TCR-binding ligand comprising contacting the TCR-displaying proteinaceous particle with a TCR-binding ligand, in the presence of and in the absence of a test compound, and determining whether the presence of the test compound reduces binding of the TCR-displaying proteinaceous particle to the TCR-binding ligand, such reduction being taken as identifying an inhibitor.

A final aspect of the invention is a method of identifying a potential inhibitor of the interaction between an TCR-displaying proteinaceous particle of the invention, and a TCR-binding ligand, for example a MHC-peptide complex, comprising contacting the TCR-displaying proteinaceous particle or TCR-binding ligand partner with a test compound and determining whether the test compound binds to the TCR-displaying proteinaceous particle and/or the TCR-binding ligand, such binding being taken as

identifying a potential inhibitor. This aspect of the invention may find particular utility in interfacial optical assays such as those carried out using the BIAcore™ system.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

Examples

The invention is further described in the following examples, which do not limit the scope of the invention in any way.

Reference is made in the following to the accompanying drawings in which:

Figures 1a and 1b show respectively the nucleic acid sequences of the α and β chains of a soluble A6 TCR, mutated so as to introduce a cysteine codon. The shading indicates the introduced cysteine codons;

Figure 2a shows the A6 TCR α chain extracellular amino acid sequence, including the $T_{48} \rightarrow C$ mutation (underlined) used to produce the novel disulphide inter-chain bond, and Figure 2b shows the A6 TCR β chain extracellular amino acid sequence, including the $S_{57} \rightarrow C$ mutation (underlined) used to produce the novel disulphide inter-chain bond;

Figure 3 Outlines the cloning of TCR α and β chains into phagmid vectors. The diagram describes a phage display vector. RSB is the ribosome-binding site. S1 or S2 are signal peptides for secretion of proteins into periplasm of *E. coli*. The * indicates translation stop codon. Either of the TCR α chain or β chain can be fused to phage coat protein, however in this diagram only TCR β chain is fused to phage coat protein.

Figure 4 details the DNA sequence of phagmid pEX746:A6.

Figure 5 expression of phage particles fusions of bacterial coat protein and dTCR in E. coli. Fusion proteins of dTCR::gIII are detected using western blotting. Phage particles are prepared from E. cloi XL-1-Blue and concentrated with PEG/NaCl. The samples are loaded in reducing or non-reducing sample buffers. Lane 1 is the sample of clone 1 containing correct sequence, and lane 2 is the sample of clone 2 containing a deletion in the α-chain encoding gene. The dsTCR::gIII fusion protein was detected at 125kDa.

Figure 6 illustrates ELISA detection of pMHC peptide complex binding activity of dTCR displayed on phage. Clone 1 binds specifically to HLA A2/Tax complex. Clone 2 can not bind to any pMHC, as no dTCR is attached to the phage particles.

Figure 7a schematic illustration of the A6 scTCR-C-Kappa DNA ribosome display construct.

Figure 7b details the complete DNA and amino acid sequences of the A6 scTCR-C-Kappa DNA ribosome display construct encoded in pUC19.

Figure 8 details the DNA sequence of pUC19-T7.

Figure 9 details the DNA sequence of the A6scTCR-C-Kappa ribosome display construct that was cloned into pUC19-T7.

Figure 10 Western blot showing the detection of in-vitro translated A6 scTCR-C-Kappa using Ambion rabbit reticulocyte lysates.

Figure 11 RT-PCR of A6 scTCR-C-Kappa mRNA on beads rescued from the ribosome display reactions.

Example 1 – Design of primers and mutagenesis of A6 Tax TCR α and β chains to introduce the cysteine residues required for the formation of a novel inter-chain disulphide bond

For mutating A6 Tax threonine 48 of exon 1 in TRAC*01 to cysteine, the following primers were designed (mutation shown in lower case):

5'-C ACA GAC AAA tgT GTG CTA GAC AT

5'-AT GTC TAG CAC Aca TTT GTC TGT G

For mutating A6 Tax serine 57 of exon 1 in both TRBC1*01 and TRBC2*01 to cysteine, the following primers were designed (mutation shown in lower case):

5'-C AGT GGG GTC tGC ACA GAC CC

5'-GG GTC TGT GCa GAC CCC ACT G

PCR mutagenesis:

Expression plasmids containing the genes for the A6 Tax TCR α or β chain were mutated using the α-chain primers or the β-chain primers respectively, as follows. 100 ng of plasmid was mixed with 5 µl 10 mM dNTP, 25 µl 10xPfu-buffer (Stratagene), 10 units Pfu polymerase (Stratagene) and the final volume was adjusted to 240 µl with H_2O. 48 µl of this mix was supplemented with primers diluted to give a final concentration of 0.2 µM in 50 µl final reaction volume. After an initial denaturation step of 30 seconds at 95°C, the reaction mixture was subjected to 15 rounds of denaturation (95°C, 30 sec.), annealing (55°C, 60 sec.), and elongation (73°C, 8 min.) in a Hybaid PCR express PCR machine. The product was then digested for 5 hours at 37°C with 10 units of DpnI restriction enzyme (New England Biolabs). 10 µl of the digested reaction was transformed into competent XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l

K_2HPO_4, 100 mg/l Ampicillin). Plasmid DNA was purified on a Qiagen mini-prep column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University. The respective mutated nucleic acid and amino acid sequences are shown in Figures 1a and 2a for the α chain and Figures 1b and 2b for the β chain.

Example 2 - Construction of phage display vectors and cloning of α and β chains into the phagemid vectors.

In order to display dTCR on filamentous phage particles, phagemid vectors were constructed for expression of fusion proteins comprising dTCR with a phage coat protein. These vectors contain a pUC19 origin, an M13 origin, a bla (Ampicillin resistant) gene, Lac promoter/operator and a CAP-binding site. The design of these vectors is outlined in Figure 3., which describes vectors encoding for both dTCR β chain-gp3 or dTCR β chain-gp8 fusion proteins in addition to a soluble TCR α chain. The expression vectors containing the DNA sequences of the mutated A6 TCR α and β chains incorporating the additional cysteine residues required for the formation of a novel disulphide prepared in Example 1 and as shown in figures 1a and 1b were used as the source of the A6 TCR α and β chains for the production of a phagemid encoding this TCR. The complete DNA sequence of the phagemid construct utilised is given in Figure 4.

The molecular cloning methods for constructing the vectors are described in "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell". Primers listed in table-1 are used for construction of the vectors. A example of the PCR programme is 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 5 seconds, 53°C for 5 seconds and 72°C for 90 seconds, followed by 1 cycles of 72°C for 10 minutes, and then hold at 4°C. The hifidelity Taq DNA polymerase is purchased from Roche.

Table 1. Primers used for construction of dTCR phage display vectors

Primer name	Sequence 5′ to 3′	Usage
YOL1	TAATAATACGTATAATAATATTCTATTTCAAG GAGACAGTC	pEX746 construction, SnaB1/ clone SDII
YOL2	CAATCCAGCGGCTGCCGTAGGCAATAGGTATT TCATTATGACTGTCTCCTTGAAATAG	pEX746 construction, clone s1
YOL3	CtaCGGCAGCCGCTGGATTGTTATTACTCGCG GCCCAGCCGGCCATGGCccag	Clone S1
YOL4	GTTCTGCTCCACTTCCTTCTGGGCCATGGCCG GCTGGGCCG	Fuse S1 and A6 Vα
YOL5	CAGAAGGAAGTGGAGCAGAAC	Clone A6 Vα
YOL6	CTTCTTAAAGAATTCTTAATTAACCTAGGTTA TTAGGAACTTTCTGGGCTGGGGAAG	Clone A6 Cα
YOL7	GTTAATTAAGAATTCTTTAAGAAGGAGATATA CATATGAAAAAATTATTATTCGCAATTC	Clone SDIII
YOL8	CGCGCTGTGAGAATAGAAAGGAACAACTAAAG GAATTGCGAATAATAATTTTTTCATATG	Clone S2
YOL9	CTTTCTATTCTCACAGCGCGCAGGCTGGTGTC ACTCAGAC	Clone S2 and A6 Vβ
YOL10	ATGATGTCTAGATGCGGCCGCGTCTGCTCTAC CCCAGGCCTC	Clone A6 Cβ
YOL11	GCATCTAGACATCATCACCATCATCACTAGAC TGTTGAAAGTTGTTTAGCAAAAC	Clone $(His)_6$ tag and amber codon and fuse to gIII
YOL12	CTAGAGGGTACCTTATTAAGACTCCTTATTAC GCAGTATG	Clone gIII

Example 3 - Expression of fusions of bacterial coat protein and dTCR in E. coli.

In order to validate the constructs made in Example 2, phage particles displaying dTCR are prepared using methods described previously for the generation of phage particles displaying antibody scFvs (Li *et al*, 2000, Journal of Immunological Methods **236:** 133-146) with the following modifications. E. coli XL-1-Blue cells containing pEX746:A6 phagemid were used to inoculate 5 ml of LBatg (Lennox L broth containing 100μg/ml of ampicillin, 12.5 μg/ml tetracycline and 2% glucose), and then

the culture was incubated with shaking at 37°C overnight (16 hours). 50 µl of the overnight culture was used to inoculate 5 ml of TYPatg (TYP is 16g/l of peptone, 16g/l of yeast extract, 5g/l of NaCl and 2.5g/l of K_2HPO_4), and then the culture was incubated with shaking at 37°C until $OD_{600nm} = 0.8$. Helper phage M13 K07 was added to the culture to the final concentration of 5×10^9 pfu/ml. The culture was then incubated at 37°C stationary for thirty minutes and then with shaking at 200 rpm for further 30 minutes. The medium of above culture was then changed to TYPak (TYP containing 100µg/ml of ampicillin, 25 µg/ml of kanamycin), the culture was then incubated at 25°C with shaking at 250 rpm for 36 to 48 hours. The culture was then centrifuged at 4°C for 30 minutes at 4000 rpm. The supernatant was filtrated through a 0.45 µm syringe filter and stored at 4°C for further concentration or analysis.

The fusion protein of filamentous coat protein and dsTCR was detected by western blotting. Approximately 10^{11} cfu phage particles were loaded on each lane of an SDS-PAGE gel in both reducing and non-reducing loading buffer. Separated proteins were probed primary with an anti-M13 gIII mAb, followed by a second antibody conjugated with Horseradish Peroxidase (HRP). The HRP activity was then detected with Opti-4CN substrate kit from Bio-Rad (Figure 5). The data indicated that disulphide-bonded TCR of clone 1 is fused with filamentous phage coat protein, gIII protein.

Example 4 - Detection of functional dTCR on filamentous phage particles

The presence of functional (cognate peptide-MHC binding) dTCR displayed on the phage particles was detected using a phage ELISA method.

TCR-Phage ELISA

Binding of the TCR-dispalying phage particles to immobilised peptide-MHC in ELISA is detected with primary rabbit anti-fd antisera (Sigma) followed by alkaline phosphatase (AP) conjugated anti-Rabbit mAb (Sigma). Non specific protein binding sites in the plates can be blocked with 2% MPBS or 3% BSA-PBS

Materials and reagents

1. Coating buffer, PBS
2. PBS: 138mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4
3. MPBS , 3% marvel-PBS
4. PBS-Tween: PBS, 0.1% Tween-20
5. Substrate solution, Sigma FAST pNPP, Cat# N2770

Method

1. Rinse NeutrAvidin coated wells twice with PBS.
2. Add 25µl of biotin-HLA A2 Tax or biotin-HLA A2 NYESO in PBS at concentration of 10 µg/ml, and incubate at room temperature for 30 to 60 min.
3. Rinse the wells twice with PBS
4. Add 300 µl of 3% Marvel-PBS, and incubate at room temperature for 1hr. Mix the TCR-phage suspension with 1 volume of 3% Marvel-PBS and incubate at room temperature.
5. Rinse the wells twice with PBS
6. Add 25 µl of the mixture of phage-TCR/Marvel-PBS, incubate on ice for 1hr
7. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.
8. Add 25 µl of ice cold rabbit anti-fd antibody diluted 1:1000 in Marvel-PBS, and incubate on ice for 1hr
9. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.
10. Add 25 µl of ice cold anti-rabbit mAb-Ap conjugate diluted 1:50,000 in Marvel-PBS, and incubate on ice for 1hr
11. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.
12. Add 150 µl of Alkaline phosphatase yellow to each well and read the signal at 405nm

The results presented in Figure 6 indicate clone 1 produced a phage particle displaying a dTCR which can bind specifically to its cognate pMHC.

Example 5 – scTCR ribosome display

Construction of Ribosome display scTCR vectors for use in generation of ribosome display PCR templates.

Ribosome display constructs were cloned into the readily available DNA plasmid pUC19 in order to generate an error free and stable DNA PCR template from which to conduct subsequent ribosome display experiments. Vector construction was undertaken in two steps so as to avoid the use of large oligonucleotide primers (with their associated error problems). The final A6 scTCR-C-Kappa DNA ribosome display construct is shown in a schematic form in figure 7a and both DNA and protein sequences are shown in Figure 7b. This construct can be excised from pUC19 as a Pst1/EcoR1 double digest.

The molecular cloning methods for constructing the vectors are described in "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell". Primers listed in Table 2 are used for construction of the vectors. The PCR programme utilised was as follows - 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 30 seconds, 55°C for 20 seconds and 72°C for 120 seconds, followed by 1 cycles of 72°C for 5 minutes, and then hold at 4°C. The Pfu DNA polymerase is purchased from Strategene. Oligonucleotide primers used are described in table 2.

Construction of pUC19-T7- Step 1

The construction of pUC19-T7 is described below, the construction results in a pUC19 vector containing a T7 promoter region followed by a short space region and the an optimum eukaryotic Kozak sequence. This is an essential part of the ribosome display construct as it is required for the initiation of transcription of any attached sequence in rabbit retucleocyte lysates. Sequences for ribosome display such as the A6scTCR-

Ckappa can be ligated into the pUC19-T7 vector between the Nco1 and EcoR1 restriction sites.

Equimolar amounts of the primer Rev-link and For-link were annealed by heating to 94°C for 10 min and slowly cooling the reaction to room temperature. This results in the formation of a double stranded DNA complex that can be seen below.

```
5'AGCTGCAGCTAATACGACTCACTATAGGAACAGGCCACCATGG
          CGTCGATTATGCTGAGTGATATCCTTGTCCGGTGGTACCCTAG 3'
```

The 5' region contains an overhanging sticky end complimentary to a HindIII restriction site whilst the 3' end contains a sticky end that is complimentary to a BamH1 restriction site.

The annealed oligonucleotides were ligated into Hind III/BamHI double-digested pUC19 which had been purified by agarose gel electrophoresis, excised and further purified with the Qiagen gel extraction kit. The ligations were transformed into *E. coli* XL1-BLUE. Individual pUC19-T7 clones were sequenced to confirm the presence of the correct sequence. The sequence is shown in Figure 8.

Construction of A6scTCR-C-Kappa vector - Sstep 2.

Construction of the single chain A6scTCR-C-Kappa DNA sequence requires the generation of three PCR fragments that must then be assembled into one A6scTCR-C-Kappa fragment. The fragments consist of (a.) the A6 TCR alpha chain variable region flanked by a Nco1 site in the 5' region and a section of Glycine Serine linker in the 3' region flanked by a BamH1 restriction site. This product was generated via a standard PCR of the vector pEX202 with the primers 45 and 50 (See Table 2). Fragment (b.) A6 TCR beta variable and constant region flanked by a BamH1 restriction site in the 5' region followed by a section of Glycine Serine linker. This product was generated via a standard PCR of the vector pEX207 with the primers 72 and 73 (See Table 2).

Fragment (c.) Portion of a human C-kappa region generated by a standard PCR of the p147 vector with the primers 61-60 (See Table 2). All PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit.

Fragments (b.) and (c.) were fused by a standard overlap PCR via the complementarity in their primer sequences 73 and 61(See Table 2). The PCR was carried out via the primers 72 and 60 (See Table 2). The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit. This fragment is termed (d.).

Fragment (a.) was double digested with Nco1 and BamH1 whilst fragment (d.) was double digested with BamH1 and EcoR1. pUC19-T7 was double digested with Nco1 and EcoR1. All digested DNA products were run on a 1.2% TBE agarose gel and DNA bands of the correct size were excised and purified using the Qiagen gel extraction kit. The digested pUC19-T7, fragments (a.) and (d.) were ligated and transformed into E. coli XL1-BLUE. Transformants were sequenced to confirm the correct sequence. The sequence of the A6scTCR-C-Kappa ribosome display construct that was cloned into pUC19 is shown in Figure 9 flanked by its Pst1 and EcoR1 sites.

Table 2.

Oligonucleotides used (Purchased from MWG).

Rev-Link	5'GATCCCATGGTGGCCTGTTCCTATAGTGAGTCGTATTAGCTGC
For-Link	5'AGCTGCAGCTAATACGACTCACTATAGGAACAGGCCACCATGG
45-A6	5'CCACCATGGGCCAGAAGGAAGTGGAGCAGAACTC
7 A6-Beta(RT-PCR)(a)	5'CGAGAGCCCGTAGAACTGGACTTG
49-A6-BamH1-F	5'GTGGATCCGGCGGTGGCGGGTCGAACGCTGGTGTCA CTCAGACCCC
50-A6-BamH1-R	5'CCGGATCCACCTCCGCCTGAACCGCCTCCACCGGTGACCACAAC CTGGGTCCCTG
60-Kappa-rev-	5' CTGAGAATTCTTATGACTCTCCGCGGTTGAAGCTC

EcoR1	
61-Betac-Kappa-for1	**5'** TGACGAATTCTGACTCTCCGCGGTTGAAGCTC
71 T7-Primer	**5'** AGCTGCAGCTAATACGACTCACTATAGG
72 A6-beta	**5'** GGCCACCATGGGCAACGCTGGTGTCACTCAGACCCC
73-A6-cons-rev	5' TGAACCGCCTCCACCGTCTGCTCTACCCCAGGCCTCGGCG
75 Kappa-rev	5' TGACTCTCCGCGGTTGAAGCTC

Demonstration of the production of A6scTCR-C-Kappa by In Vitro transcription translation.

Preparation of A6scTCR-C-Kappa PCR product for In Vitro transcription translation.

Here we describe the synthesis of A6scTCR-C-Kappa via In Vitro transcription translation in the presence of biotinylated lysine and its subsequent detection by western blotting and detection with alkaline phosphatase labelled streptavidin.

The A6scTCR-C-Kappa PCR product was prepared in a standard PCR reaction using the vector A6scTCR-C-Kappa as template and PCR primers 71 and 60. Primer 60 contains a stop codon to allow the release of the scTCR from the ribosome. Pfu polymerase (Strategene) was used for increased fidelity during PCR synthesis. The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit.

The transcription translation reactions were carried out using the Ambion PROTEINscript II Linked transcription translation kit Cat 1280-1287 with 300ng of the above described PCR product. Three transcription translation reactions were set up according to the manufactures protocol. The one modification was the addition of biotinylated lysine from the Transcend TM Non-Radioactive Translation Detection System.

Reaction 1 A6scTCR-C-Kappa 300ng with 2µl biotinylated lysine

Reaction 2 A6scTCR-C-Kappa 300ng without 2µl biotinylated lysine

reaction 3 No DNA control with 2µl biotinylated lysine.

Two microliters of each reaction was run on a 4-20% Novex gradient SDS-PAGE gel (Invitrogen). Additionaly a number of dilutions of a control biotinylated TCR were also run. The gel was blotted and the proteins detected with streptavidin alkaline phosphatase and subsequently colometrically developed with Western Blue ® Stabilized Substrate for Alkaline Phosphatase as described in the Transcend TM Non-Radioactive Translation Detection System protocol. The western blot is shown in Figure 10.

In the no DNA control and A6scTCR-C-Kappa reaction without biotinylated lysine no band of approximately the correct size can be seen as expected whilst in the A6scTCR-C-Kappa reaction in the presence of biotinylated lysine a band of approximately the correct size can be seen. This demonstrates the synthesis of the A6scTCR-C-Kappa TCR by *In Vitro* transcription translation.

Preparation of A6scTCR-C-Kappa ribosome display PCR product.

The A6scTCR-C-Kappa PCR product was prepared in a standard PCR reaction using the vector A6scTCR-C-Kappa as template and PCR primers 71 and 75 (See Table 2). Primer 75 does not contain a stop codon. Pfu polymerase (Strategene) was used for increased fidelity during PCR synthesis. The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size were excised and purified using the Qiagen gel extraction kit.

Ribosome Display Process

Transcription and translation of A6scTCR-C-Kappa

The transcription / translation reactions were carried out using the Ambion PROTEINscript II Linked transcription translation kit (Cat No. 1280-1287)

Transcription reactions

The following transcription reactions were set up in Ambion 0.5 ml non stick tubes (Cat No. 12350).

Contents	tube 1 (Normal A6)	2 (Control)
Water	4.53 µl	5.7µl
Template (PCR product)	**A6scTCR-C-Kappa PCR product 1.17µl (300 ng)**	No DNA
5X transcription mix	2 µl	2µl
Enzyme mix	2µl	2µl
Superasin RNase inhibitor ambion	0.3µl	0.3µl
Final volume	10µl	10µl

The tubes were incubated at 30°C for 60 min on a PCR block with the hot lid off.

Translation reactions

The following translation reactions were set up in Ambion 0.5 ml non stick tubes.

Contents	1 (Normal A6)	2 (Control)
Reticulocyte Lysate	105µl	105 µl
25mM Mg-Acetate	3µl	3 µl
Translation Mix	7.5µl	7.5 µl
Methionine	7.5µl	7.5 µl
Water	18µl	18 µl
Superasin RNase inhibitor	3µl	3 µl
Transcription reaction	6µl tube 1 above	6µl tube 2 above

Each tube contains enough for 3x50µl selections. The tubes were mixed and incubated at 30°C for 60 min on a PCR block with the hot lid off. After 30 min 3 Unit of RQ1 RNase free DNase (Promega) was added to destroy the original DNA template in tube 1 and 3 Unit RQ1 RNase free DNase (Promega) in tube 2. After 60 min 18µl of

Heparin solution was added to translation reaction 2 and 18µl of Heparin solution was added to translation reaction 1. Samples were stored on ice ready for selection against HLA-coated beads.

Coating of magnetic beads.

20µl of resuspended Streptavidin Magnetic Particles (Roche Cat. No. 1641778) were transferred into a sterile RNase free 1.5 ml eppendorf tube. The beads were immobilised with a Magnetic Particle Separator (Roche Cat. No. 1641794) and the supernatant was removed. The beads were then washed with 100 µl of RNase free 1 X PBS (10 x PBS Ambion Cat No. 9624, Ambion H_2O Cat No. 9930) the beads were immobilised and the supernatant was removed. A total of 3 PBS washes were carried out.

The beads were resuspended in 20µl of PBS and the contents split evenly between two tubes (10µl each). One tube will be used to produce control blocked beads and the other tubes to produce HLA A2-TAX coated beads.

To the control beads tube 80µl of BSA/Biotin solution was added and mixed. The BSA/Biotin solution was made up as follows. 10µl of a 0.2M Tris base 0.1M Biotin solution was added to 990µl of PBS 0.1 % BSA(Ambion Ultrapure Cat No. 2616). Also 20 µl of Heparin solution (138 mg/ml Heparin (Sigma H-3393) in 1 x PBS) was added and the solution mixed. The beads were incubated at room temperature for 1 hour with intermittent mixing. The beads were then washed three times with 100µl of PBS and were resuspended in 10 µl of PBS, 0.1% BSA.

The HLA-TAX coated beads were prepared as follows. 40 µl of HLA-TAX (1.15 mg/ml prepared as described in WO99/60120) was added to the 10 µl of beads and mixed. The beads were incubated at room temperature for 15 min and then 20 µl of BSA 50mg/ml Ambion Cat 2616 and 20 µl of heparin solution (see above) were added and mixed. The beads were incubated for a further 45 min and then 20 µl of

BSA/Biotin solution was added. The beads were then washed three times with 100µl of PBS and were re-suspended in 10 µl of PBS, 0.1% BSA.

Panning with magnetic beads

The A6 scTCR translation reaction was split into three 50µl aliquots and each aliquot received either 2µl of the following beads:

Control (no HLA)

HLA-A2-TAX

HLA-A2-TAX plus 10µg soluble A6scTCR

A control translation reaction was also carried out and split into three 50µl aliquots and each aliquot received either 2µl of the following beads

Control (no HLA)

HLA-A2-TAX

HLA-A2-TAX plus 10µg soluble A6scTCR

This gave a total of six tubes. The tubes were incubated on a PCR block at 5°C for 60 min with intermittent mixing.

The beads were then washed three times with 100µl ice cold buffer (PBS, 5mM Mg-acetate, 0.2% Tween 20(Sigma RNase free). Each aliquot of beads were then re-suspended in 50µl of 1 x RQ1 DNase digestion buffer containing 1µl (40 U) of Superasin and 1µl (1U) of RQ1 DNase. The beads were incubated on a PCR block for 30 min at 30°C.

The beads were then washed three times with 100µl ice cold buffer (PBS, 5mM Mg-acetate, 0.2% Tween 20) and once with ice cold H_2O. The beads were re-suspended in 10µl of RNase free H_2O. The beads were then frozen ready for RT-PCR.

RT-PCR of A6 scTCR-C-Kappa mRNA on beads rescued from the ribosome display reactions.

The RT PCR reactions on the beads were carried out using the Titan one tube RT-PCR kit cat 1855476 as described in the manufacturers protocols. Two microliters of beads were added into each RT-PCR reaction along with the primers 45 and 7 and 0.3µl of Superasin RNase inhibitor.

For each RT-PCR reaction a second PCR only reaction was set up which differed only by the fact that no reverse transcriptase was present just Roche high fidelity polymerase. This second reaction served as a control for DNA contamination. Additionally a RT-PCR positive control control was set up using 1ng of the vector A6scTCR-C-Kappa.

The reactions were cycled as follows. An RT-PCR step was carried out by incubation of the samples at 50°C for 30 min followed by the inactivation of the reverse transcriptase by incubation at 94°C for 3 min on a PCR block.

The reactions were then PCR cycles as follows for a total of 38 cycles.

94°C 30 seconds

55°C 20 seconds

68°C 130 seconds.

The PCR reaction was finished by incubation at 72°C for 4 minutes.

Great care was taken during all ribosome display steps to avoid RNase contamination.

The RT-PCR and PCR reactions were run on a 1.6% TBE agarose gel which can be seen in Figure 11. Analysis of the gel shows that there is no DNA contamination and that all PCR products are derived from mRNA. The DNA band of the correct size in lane 2 demonstrates that ribosome displayed A6scTCR-C-Kappa was selected out by HLA-A2-TAX coated beads. Lane 3 shows that we can inhibit this specific selection of ribosome displayed A6scTCR-C-Kappa by the addition of soluble A6scTCR. The significant reduction in the band intensity in lane 3 relative to the uninhibited sample

in lane 2 demonstrates this. No binding of ribosome displayed A6scTCR-C-Kappa could be shown against control non-HLA coated beads.

Claims:

1. A proteinaceous particle displaying on its surface a T-cell receptor (TCR), characterised in that

(i) the proteinaceous particle is a ribosome and the TCR is a single chain TCR (scTCR) polypeptide, or

(ii) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a human scTCR or a human dimeric T-cell receptor (dTCR) polypeptide pair, or

(iii) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a non-human dTCR polypeptide pair, or

(iv) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a scTCR polypeptide comprising TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain, and a disulfide bond which has no equivalent in native T cell receptors links residues of the constant region sequences.

2. A proteinaceous particle, displaying on its surface a dimeric T-cell receptor (dTCR) polypeptide pair, or a single chain T-cell receptor (scTCR) polypeptide wherein

the dTCR polypeptide pair is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains, and the scTCR is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain;

the variable region sequences of the dTCR polypeptide pair or scTCR polypeptide are mutually orientated substantially as in native TCRs; and

in the case of the scTCR polypeptide a disulfide bond which has no equivalent in native T cell receptors links residues of the polypeptide.

3. A proteinaceous particle as claimed in claim 1 or claim 2, which is a phage particle.

4. A proteinaceous particle as claimed in claim 1 or claim 2, which is a cell surface protein or polypeptide.

5. A proteinaceous particle as claimed in claim 1 or claim 2, which is a ribosome.

6. A proteinaceous particle as claimed in any of the preceding claims wherein the C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, is linked by a peptide bond to a surface exposed residue of the proteinaceous particle.

7. A proteinaceous particle as claimed in any of claims 1 to 4 wherein the C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the

scTCR polypeptide, is linked by a disulfide bond to a surface exposed cysteine residue of the proteinaceous particle.

8. A proteinaceous particle as claimed in any of the preceding claims wherein an scTCR polypeptide is displayed which comprises

a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region

a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR β chain constant region extracellular sequence, and

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment.

9. A proteinaceous particle as claimed in any of claims 1 to 7 wherein an scTCR polypeptide is displayed which comprises

a first segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region

a second segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant region extracellular sequence, and

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment.

10. A proteinaceous particle as claimed in any of claims 1 to 7 wherein a scTCR polypeptide is displayed which has

a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant region extracellular sequence,

a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region fused to the N terminus of an amino acid sequence corresponding to TCR β chain constant region extracellular sequence,

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment, or vice versa, and

a disulfide bond between the first and second chains, said disulfide bond being one which has no equivalent in native αβ or γδ T cell receptors,

the length of the linker sequence and the position of the disulfide bond being such that the variable region sequences of the first and second segments are mutually orientated substantially as in native αβ or γδ T cell receptors.

11. A proteinaceous particle as claimed in claim 10 wherein the linker sequence has the formula -P-AA-P- wherein P is proline and AA represents an amino acid sequence wherein the amino acids are glycine and serine.

12. A proteinaceous particle as claimed in claim 10 or claim 11 wherein the linker sequence links the C terminus of the first segment to the N terminus of the second segment.

13. A proteinaceous particle as claimed in claim 12 wherein the linker sequence consists of from 26 to 41 amino acids.

14. A proteinaceous particle as claimed in claim 13 wherein the linker sequence consists of 29, 30, 31 or 32 amino acids.

15. A proteinaceous particle as claimed in claim 13 wherein the linker sequence consists of 33, 34, 35 or 36 amino acids.

16. A proteinaceous particle as claimed in claim 13 wherein the linker sequence has the formula -PGGG-$(SGGGG)_5$-P- wherein P is proline, G is glycine and S is serine.

17. A proteinaceous particle as claimed in claim 13 wherein the linker sequence has the formula -PGGG-$(SGGGG)_6$-P- wherein P is proline, G is glycine and S is serine.

18. A proteinaceous particle as claimed in any of claims 1 to 4, 6 or 7 wherein a dTCR polypeptide pair is displayed which is constituted by

a first polypeptide wherein a sequence corresponding to a TCR α or δ chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant region extracellular sequence, and

a second polypeptide wherein a sequence corresponding to a TCR β or γ chain variable region sequence fused to the N terminus a sequence corresponding to a TCR β chain constant region extracellular sequence,

the first and second polypeptides being linked by a disulfide bond which has no equivalent in native $\alpha\beta$ or $\gamma\delta$ T cell receptors.

19. A proteinaceous particle as claimed in any preceding claim wherein the displayed dTCR polypeptide pair or scTCR polypeptide have amino acid sequences corresponding to αβ TCR extracellular constant and variable region sequences.

20. A proteinaceous particle as claimed in any of claims 1 to 18 wherein the displayed dTCR polypeptide pair or scTCR polypeptide have amino acid sequences corresponding to extracellular αβ TCR constant region sequences and γδ TCR variable region sequences.

21. A proteinaceous particle as claimed in any preceding claim wherein the displayed dTCR polypeptide pair or scTCR polypeptide have amino acid sequences corresponding to non-human extracellular αβ TCR constant region sequences and human TCR variable region sequences.

22. A proteinaceous particle as claimed in any of the preceding claims wherein an amino acid sequence of one member of the displayed dTCR polypeptide pair, or an amino acid sequence of the displayed scTCR, corresponds to a native TCR extracellular constant chain Ig domain sequence.

23. A proteinaceous particle as claimed in any of the preceding claims wherein the displayed dTCR polypeptide pair or displayed scTCR, includes sequences corresponding to native TCR extracellular constant chain Ig domain sequences.

24. A proteinaceous particle as claimed in claim 23 wherein a disulfide bond links amino acid residues of the said constant chain Ig domain sequences, which disulfide bond has no equivalent in native TCRs.

25. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues corresponding to amino acid residues whose β carbon atoms are less than 0.6 nm apart in native TCRs.

26. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Thr 48 of exon 1 of TRAC*01 and Ser 57 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.

27. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Thr 45 of exon 1 of TRAC*01 and Ser 77 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.

28. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Tyr 10 of exon 1 of TRAC*01 and Ser 17 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.

29. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Thr 45 of exon 1 of TRAC*01 and Asp 59 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.

30. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Ser 15 of exon 1 of TRAC*01 and Glu 15 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.

31. A proteinaceous particle as claimed in any of claims 23 to 30 wherein the sequences corresponding to native TCR extracellular constant chain Ig domain sequences are truncated at their C-termini relative to said native sequences such that the cysteine residues which form the native interchain disulphide bond are excluded.

32. A proteinaceous particle as claimed in any of claims 23 to 30 wherein in the sequences corresponding to native TCR extracellular constant chain Ig domain sequences the cysteine residues which form the native interchain disulphide bond are substituted by non-cysteine residues.

33. A proteinaceous particle as claimed in claim 32 wherein the cysteine residues which form the native interchain disulfide bond are substituted by serine or alanine.

34. A proteinaceous particle as claimed in any of the preceding claims wherein in the displayed dTCR or scTCR there is no unpaired cysteine residue corresponding an unpaired cysteine residue present in a native TCR.

35. A proteinaceous particle as claimed in any of claims 23 to 34 wherein the sequences corresponding to native TCR extracellular constant chain Ig domain sequences are truncated N-terminal to residues corresponding to those which form the non-native interchain disulphide bond.

36. A diverse library of dTCR polypeptide pairs or scTCRs polypeptides displayed on proteinaceous particles said dTCR polypeptide pairs or scTCR polypeptides having the structural features defined in any of claims 8 to 35.

37. A composition comprising (i) nucleic acid encoding (a) one chain of a dTCR polypeptide pair and (b) the other chain of a dTCR polypeptide pair fused to a nucleic acid sequence encoding a particle forming protein or (ii) nucleic acid encoding a scTCR polypeptide fused to a nucleic acid sequence encoding a particle forming protein, the dTCR pair or scTCR having the structural features defined in any of claims 8 to 35.

38. Plasmid nucleic acid sequence or sequences which comprise the nucleic acid sequence or sequences as claimed in claim 37.

39. A phagemid or phage genome nucleic acid sequence or sequences which comprises the nucleic acid sequence or sequences as claimed in claims 37.

40. A phagemid or phage genome nucleic acid sequence or sequences as claimed in claim 40 wherein non-TCR nucleic acid sequences are derived from a filamentous phage.

41. A phagemid or phage genome nucleic acid sequence composition as claimed in claim 40 wherein the said non-TCR nucleic acid sequence encodes bacteriophage 4GIII or GVIII coat proteins.

42. Nucleic acid comprising phagemid or phage genome sequence or sequences as claimed in any of claims 39 to 41 and a helper phage.

43. Nucleic acid as claimed in claim 42 wherein the helper phage is M13K07 or VCS M13 filamentous phage

44. A host cell comprising nucleic acid as claimed in any of claims 37 to 43.

45. An in-vivo expression system comprising nucleic acid as claimed in any of claims 37 to 43.

46. A method for the identification of TCRs with a specific characteristic, said method comprising subjecting a diverse library of TCRs displayed on proteinaceous particles as claimed in claim 36 to an assay which measures said characteristic, identifying those proteinaceous particles which display a TCR with the desired characteristic and isolating these proteinaceous particles.

47. A method as claimed in claim 46 wherein the specific characteristic is increased affinity for a TCR ligand.

48. A method for detecting TCR ligand complexes, which comprises:

(i) providing a TCR-displaying proteinaceous particle as claimed in any of claims 1 to 36;

(ii) contacting said TCR-displaying proteinaceous particle with a putative ligand complex; and

(iii) detecting binding of the said TCR-displaying proteinaceous particle to the putative ligand complexes

49 A method as claimed in claim 48 wherein the putative TCR ligand complexes are peptide-MHC complexes.

50. A method of identifying an inhibitor of the interaction between a TCR-displaying proteinaceous particle as claimed in any one of claims 1 to 36, and a TCR-binding ligand comprising contacting the TCR-displaying proteinaceous particle with a TCR-binding ligand, in the presence of and in the absence of a test compound, and determining whether the presence of the test compound reduces binding of the TCR-displaying proteinaceous particle to the TCR-binding ligand, such reduction being taken as identifying an inhibitor.

Figure 1a

atgcagaaggaagtggagcagaactctggacccctcagtgttccagagggagccattg
cctctctcaactgcacttacagtgaccgaggttcccagtccttcttctggtacagaca
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Figure 1b

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agtccagttctacgggctctcggagaatgacgagtggacccaggatagggccaaaccc
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Figure 2a

MQ
K_1EVEQNSGPL SVPEGAIASL NCTYSDRGSQ SFFWYRQYSG KSPELIMSIY
SNGDKEDGRF TAQLNKASQY VSLLIRDSQP SDSATYLCAV TTDSWGKLQF
GAGTQVVVTP DIQNPDPAVY QLRDSKSSDK SVCLFTDFDS QTNVSQSKDS
DVYITDKCVL DMRSMDFKSN SAVAWSNKSD FACANAFNNS IIPEDTFFPS
PESS* -

Figure 2b

M
N_1AGVTQTPKF QVLKTGQSMT LQCAQDMNHE YMSWYRQDPG MGLRLIHYSV
GAGITDQGEV PNGYNVSRST TEDFPLRLLS AAPSQTSVYF CASRPGLAGG
RPEQYFGPGT RLTVTEDLKN VFPPEVAVFE PSEAEISHTQ KATLVCLATG
FYPDHVELSW WVNGKEVHSG VCTDPQPLKE QPALNDSRYA LSSRLRVSAT
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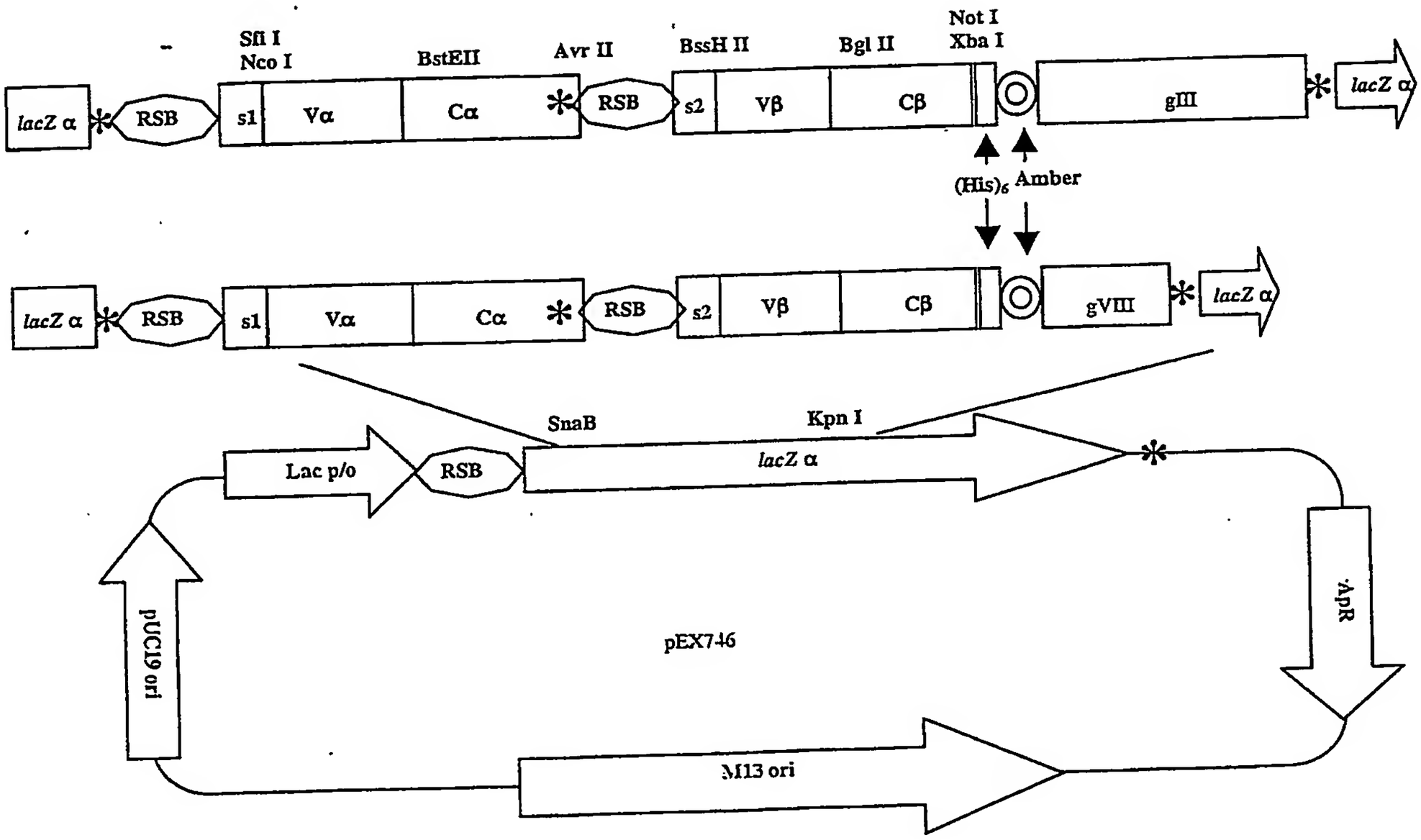
Figure 3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Nco I
BstEII
Avr II
BssH II
Bgl II
Not I
Xba I
lacZ α
RSB
s1
Vα
Cα
RSB
s2
Vβ
Cβ
gIII
lacZ α
(His)6
Amber
lacZ α
RSB
s1
Vα
Cα
RSB
s2
Vβ
Cβ
gVIII
lacZ α
SnaB
Kpn I
Lac p/o
RSB
lacZ α
pUC19 ori
pEX746
ApR
M13 ori

Figure 4

```
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  61 tttctaaata cattcaaata tgtatccgct catgagacaa taaccctgat aaatgcttca
 121 ataatattga aaaaggaaga gtatgagtat tcaacatttc cgtgtcgccc ttattccctt
 181 ttttgcggca ttttgccttc ctgtttttgc tcacccagaa acgctggtga aagtaaaaga
 241 tgctgaagat cagttgggtg cacgagtggg ttacatcgaa ctggatctca acagcggtaa
 301 gatccttgag agttttcgcc ccgaagaacg ttctccaatg atgagcactt ttaaagttct
 361 gctatgtggc gcggtattat cccgtgttga cgccgggcaa gagcaactcg gtcgccgcat
 421 acactattct cagaatgact tggttgagta ctcaccagtc acagaaaagc atcttacgga
 481 tggcatgaca gtaagagaat tatgcagtgc tgccataacc atgagtgata acactgcggc
 541 caacttactt ctgacaacga tcggaggacc gaaggagcta accgcttttt tgcacaacat
 601 gggggatcat gtaactcgcc ttgatcgttg ggaaccggag ctgaatgaag ccataccaaa
 661 cgacgagcgt gacaccacga tgcctgtagc aatggcaaca acgttgcgca aactattaac
 721 tggcgaacta cttactctag cttcccggca acaattaata gactggatgg aggcggataa
 781 agttgcagga ccacttctgc gctcggccct tccggctggc tggtttattg ctgataaatc
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 901 ctcccgtatc gtagttatct acacgacggg gagtcaggca actatggatg aacgaaatag
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2641 gtccttcttc tggtacagac aatattctgg gaaaagccct gagttgataa tgtccatata
2701 ctccaatggt gacaaagaag atggaaggtt tacagcacag ctcaataaag ccagccagta
2761 tgtttctctg ctcatcagag actcccagcc cagtgattca gccacctacc tctgtgccgt
2821 tacaactgac agctggggga aattgcagtt tggagcaggg acccaggttg tggtcacccc
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3301 tcctgaagac aggacagagc atgacactgc agtgtgccca ggatatgaac catgaataca
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4261 ctgagggtgg cggttctgag ggtggcggta ctaaacctcc tgagtacggt gatacaccta
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4801 attttgatta tgaaaagatg gcaaacgcta ataaggggggc tatgaccgaa aatgccgatg
4861 aaaacgcgct acagtctgac gctaaaggca aacttgattc tgtcgctact gattacggtg
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Figure 5

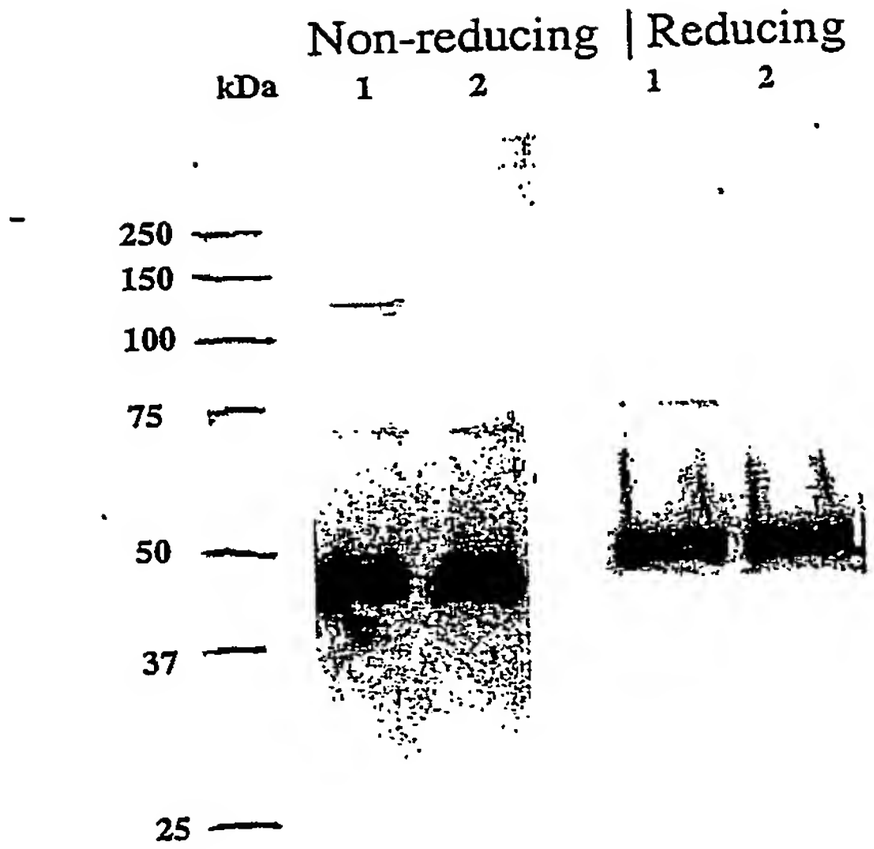
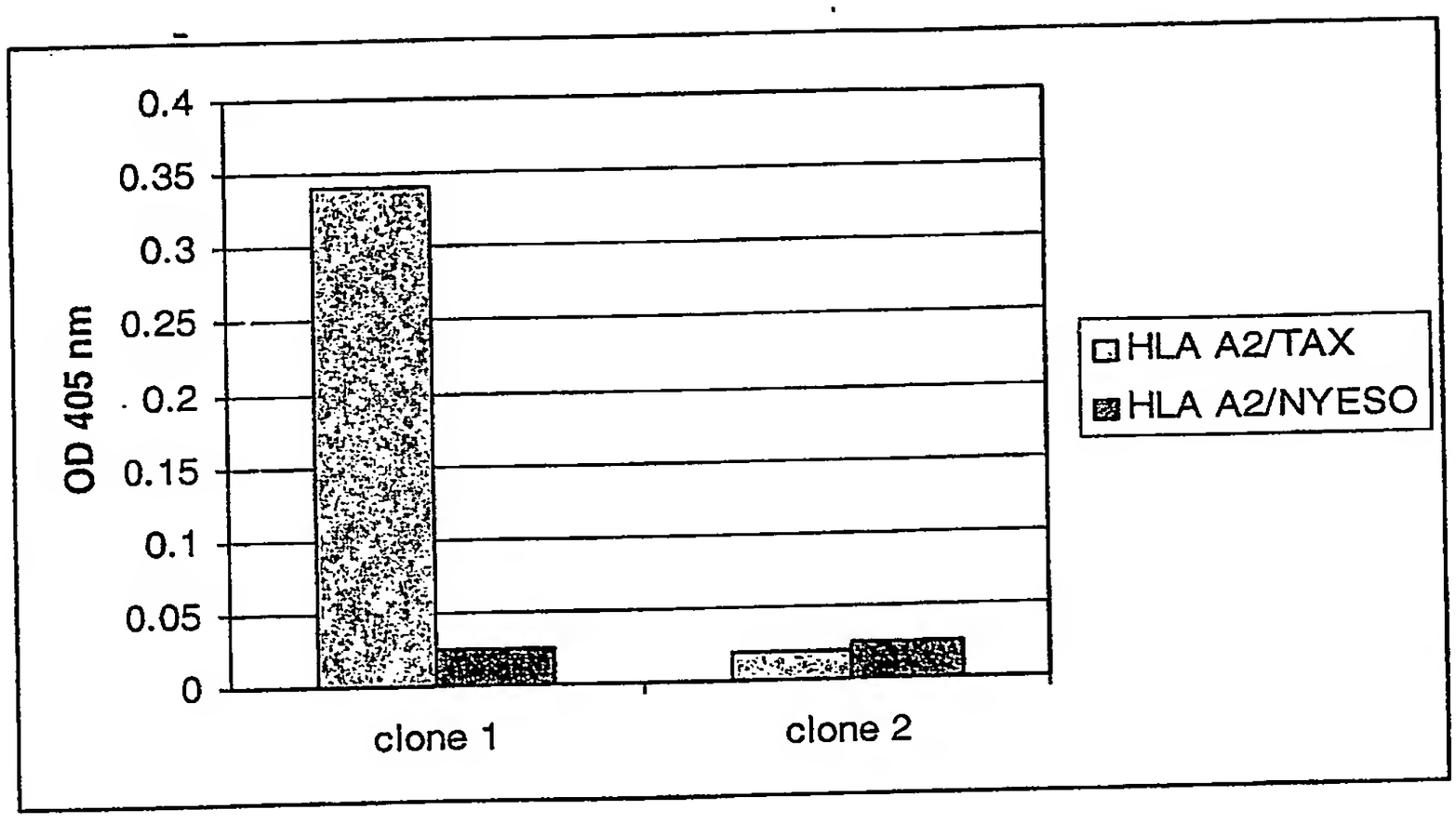


Figure 6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0.3
0.25
0.2
0.15
0.1
0.05
0
OD 405 nm
clone 1
clone 2
HLA A2/TAX
HLA A2/NYESO

Figure 7a

Schematic diagram of the A6 scTCR-C-Kappa ribosome display construct

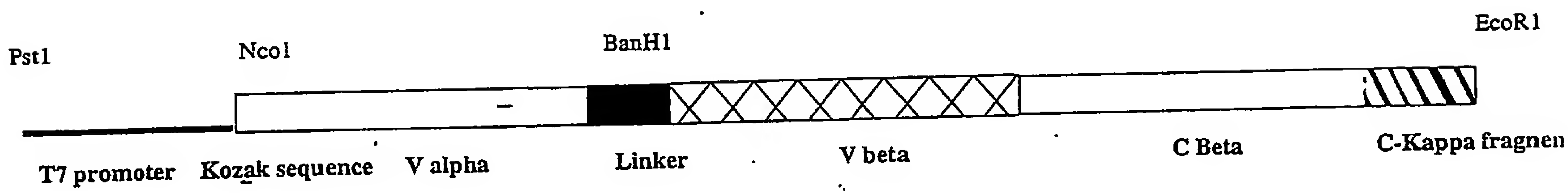

Figure 7b

```
                 T7 Promoter sequence        Kozak sequence
                                              NcoI
         PstI
                                                   M   G   Q   K ·
  1    AGCTGCAGCT AATACGACTC ACTATAGGAA CAGGCCACCA TGGGCCAGAA
       TCGACGTCGA TTATGCTGAG TGATATCCTT GTCCGGTGGT ACCCGGTCTT
       · E  V  E   Q  N  S  G   P  L  S   V  P  E   G  A  I  A ·

 51    GGAAGTGGAG CAGAACTCTG GACCCCTCAG TGTTCCAGAG GGAGCCATTG
       CCTTCACCTC GTCTTGAGAC CTGGGGAGTC ACAAGGTCTC CCTCGGTAAC
       ·  S  L  N   C  T  Y   S  D  R  G   S  Q  S   F  F  W

101    CCTCTCTCAA CTGCACTTAC AGTGACCGAG GTTCCCAGTC CTTCTTCTGG
       GGAGAGAGTT GACGTGAATG TCACTGGCTC CAAGGGTCAG GAAGAAGACC
        Y  R  Q  Y   S  G  K   S  P  E   L  I  M  S   I  Y  S ·

151    TACAGACAAT ATTCTGGGAA AAGCCCTGAG TTGATAATGT CCATATACTC
       ATGTCTGTTA TAAGACCCTT TTCGGGACTC AACTATTACA GGTATATGAG
       · N  G  D   K  E  D  G   R  F  T   A  Q  L   N  K  A  S ·

201    CAATGGTGAC AAAGAAGATG GAAGGTTTAC AGCACAGCTC AATAAAGCCA
       GTTACCACTG TTTCTTCTAC CTTCCAAATG TCGTGTCGAG TTATTTCGGT
       ·  Q  Y  V   S  L  L   I  R  D  S   Q  P  S   D  S  A

251    GCCAGTATGT TTCTCTGCTC ATCAGAGACT CCCAGCCCAG TGATTCAGCC
       CGGTCATACA AAGAGACGAG TAGTCTCTGA GGGTCGGGTC ACTAAGTCGG
                                   PvuII

        T  Y  L  C   A  V  T   T  D  S   W  G  K  L   Q  F  G ·

301    ACCTACCTCT GTGCCGTTAC AACTGACAGC TGGGGGAAAT TGCAGTTTGG
       TGGATGGAGA CACGGCAATG TTGACTGTCG ACCCCCTTTA ACGTCAAACC
                                 AgeI                     BamHI

       · A  G  T   Q  V  V  V   T  G  G   G  G  S   G  G  G  G ·

351    AGCAGGGACC CAGGTTGTGG TCACCGGTGG AGGCGGTTCA GGCGGAGGTG
```

```
                TCGTCCCTGG GTCCAACACC AGTGGCCACC TCCGCCAAGT CCGCCTCCAC
                BamHI
                ------
                .  S  G  G   G  G  S   N  A  G  V   T  Q  T   P  K  F
                ------------------------------------------------------
401             GATCCGGCGG TGGCGGGTCG AACGCTGGTG TCACTCAGAC CCCAAAATTC
                CTAGGCCGCC ACCGCCCAGC TTGCGACCAC AGTGAGTCTG GGGTTTTAAG
                                                PstI
                                                ------
                 Q  V  L  K   T  G  Q   S  M  T   L  Q  C  A   Q  D  M  .
                ------------------------------------------------------
451             CAGGTCCTGA AGACAGGACA GAGCATGACA CTGCAGTGTG CCCAGGATAT
                GTCCAGGACT TCTGTCCTGT CTCGTACTGT GACGTCACAC GGGTCCTATA
                .  N  H  E   Y  M  S  W   Y  R  Q   D  P  G   M  G  L  R  .
                ------------------------------------------------------
501             GAACCATGAA TACATGTCCT GGTATCGACA AGACCCAGGC ATGGGGCTGA
                CTTGGTACTT ATGTACAGGA CCATAGCTGT TCTGGGTCCG TACCCCGACT
                .  L  I  H   Y  S  V   G  A  G  I   T  D  Q   G  E  V
                ------------------------------------------------------
551             GGCTGATTCA TTACTCAGTT GGTGCTGGTA TCACTGACCA AGGAGAAGTC
                CCGACTAAGT AATGAGTCAA CCACGACCAT AGTGACTGGT TCCTCTTCAG
                 P  N  G  Y   N  V  S   R  S  T   T  E  D  F   P  L  R  .
                ------------------------------------------------------
601             CCCAATGGCT ACAATGTCTC CAGATCAACC ACAGAGGATT TCCCGCTCAG
                GGGTTACCGA TGTTACAGAG GTCTAGTTGG TGTCTCCTAA AGGGCGAGTC
                .  L  L  S   A  A  P  S   Q  T  S   V  Y  F   C  A  S  R  .
                ------------------------------------------------------
651             GCTGCTGTCG GCTGCTCCCT CCCAGACATC TGTGTACTTC TGTGCCAGCA
                CGACGACAGC CGACGAGGGA GGGTCTGTAG ACACATGAAG ACACGGTCGT
                                                ScaI
                                                ------
                .  P  G  L   A  G  G   R  P  E  Q   Y  F  G   P  G  T
                ------------------------------------------------------
701             GGCCGGGACT AGCGGGAGGG CGACCAGAGC AGTACTTCGG GCCGGGCACC
                CCGGCCCTGA TCGCCCTCCC GCTGGTCTCG TCATGAAGCC CGGCCCGTGG
                 R  L  T  V   T  E  D   L  K  N   V  F  P  P   E  V  A  .
                ------------------------------------------------------
751             AGGCTCACGG TCACAGAGGA CCTGAAAAAC GTGTTCCCAC CCGAGGTCGC
                TCCGAGTGCC AGTGTCTCCT GGACTTTTTG CACAAGGGTG GGCTCCAGCG
                                        BglII
                                        ------
                .  V  F  E   P  S  E   A  E  I   S  H  T  Q   K  A  T  L  .
                ------------------------------------------------------
801             TGTGTTTGAG CCATCAGAAG CAGAGATCTC CCACACCCAA AAGGCCACAC
                ACACAAACTC GGTAGTCTTC GTCTCTAGAG GGTGTGGGTT TTCCGGTGTG
                .  V  C  L   A  T  G   F  Y  P  D   H  V  E   L  S  W
                ------------------------------------------------------
851             TGGTGTGCCT GGCCACAGGC TTCTACCCCG ACCACGTGGA GCTGAGCTGG
                ACCACACGGA CCGGTGTCCG AAGATGGGGC TGGTGCACCT CGACTCGACC
                                ApaLI
                                -------
                 W  V  N  G   K  E  V   H  S  G   V  S  T  D   P  Q  P  .
                ------------------------------------------------------
901             TGGGTGAATG GGAAGGAGGT GCACAGTGGG GTCAGCACAG ACCCGCAGCC
                ACCCACTTAC CCTTCCTCCA CGTGTCACCC CAGTCGTGTC TGGGCGTCGG
                .  L  K  E   Q  P  A  L   N  D  S   R  Y  A   L  S  S  R  .
                ------------------------------------------------------
951             CCTCAAGGAG CAGCCCGCCC TCAATGACTC CAGATACGCT CTGAGCAGCC
                GGAGTTCCTC GTCGGGCGGG AGTTACTGAG GTCTATGCGA GACTCGTCGG
                 Bsu36I
                 -------
                .  L  R  V   S  A  T   F  W  Q  D   P  R  N   H  F  R
                ------------------------------------------------------
1001            GCCTGAGGGT CTCGGCCACC TTCTGGCAGG ACCCCCGCAA CCACTTCCGC
                CGGACTCCCA GAGCCGGTGG AAGACCGTCC TGGGGGCGTT GGTGAAGGCG
                 C  Q  V  Q   F  Y  G   L  S  E   N  D  E  W   T  Q  D  .
```

```
1051  TGTCAAGTCC AGTTCTACGG GCTCTCGGAG AATGACGAGT GGACCCAGGA
      ACAGTTCAGG TCAAGATGCC CGAGAGCCTC TTACTGCTCA CCTGGGTCCT
                                              StuI

      · R  A  K   P  V  T  Q   I  V  S   A  E  A   W  G  R  A ·

1101  TAGGGCCAAA CCCGTCACCC AGATCGTCAG CGCCGAGGCC TGGGGTAGAG
      ATCCCGGTTT GGGCAGTGGG TCTAGCAGTC GCGGCTCCGG ACCCCATCTC
      · D  G  G   G  G  S   L  S  S  T   L  T  L   S  K  A

1151  CAGACGGTGG AGGCGGTTCA CTCAGCAGCA CCCTGACGCT GAGCAAAGCA
      GTCTGCCACC TCCGCCAAGT GAGTCGTCGT GGGACTGCGA CTCGTTTCGT
       D  Y  E  K   H  K  V   Y  A  C   E  V  T  H   Q  G  L ·

1201  GACTACGAGA AACACAAAGT CTACGCCTGC GAAGTCACCC ATCAGGGCCT
      CTGATGCTCT TTGTGTTTCA GATGCGGACG CTTCAGTGGG TAGTCCCGGA
                                                   EcoRI

      · S  S  P   V  T  K  S   F  N  R   G  E  S

1251  GAGTTCGCCC GTCACAAAGA GCTTCAACCG CGGAGAGTCA TAAGAATTCT
      CTCAAGCGGG CAGTGTTTCT CGAAGTTGGC GCCTCTCAGT ATTCTTAAGA
1301  CAG
      GTC
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Figure 8

pUC19-T7 sequence

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 141 ataggcgagt actctgttat tgggactatt tacgaagtta ttataacttt ttccttctca tactcataag
 211 ttgtaaaggc acagcgggaa taagggaaaa aacgccgtaa aacggaagga caaaaacgag tgggtctttg
 281 cgaccacttt cattttctac gacttctagt caacccacgt gctcacccaa tgtagcttga cctagagttg
 351 tcgccattct aggaactctc aaaagcgggg cttcttgcaa aaggttacta ctcgtgaaaa tttcaagacg
 421 atacaccgcg ccataatagg gcataactgc ggcccgttct cgttgagcca gcggcgtatg tgataagagt
 491 cttactgaac caactcatga gtggtcagtg tcttttcgta gaatgcctac cgtactgtca ttctcttaat
 561 acgtcacgac ggtattggta ctcactattg tgacgccggt tgaatgaaga ctgttgctag cctcctggct
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 701 cttacttcgg tatggtttgc tgctcgcact gtggtgctac ggacatcgtt accgttgttg caacgcgttt
 771 gataattgac cgcttgatga atgagatcga agggccgttg ttaattatct gacctacctc cgcctatttc
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1331 aaaaggcttc cattgaccga agtcgtctcg cgtctatggt ttatgacagg aagatcacat cggcatcaat
1401 ccggtggtga agttcttgag acatcgtggc ggatgtatgg agcgagacga ttaggacaat ggtcaccgac
1471 gacggtcacc gctattcagc acagaatggc ccaacctgag ttctgctatc aatggcctat tccgcgtcgc
1541 cagcccgact tgccccccaa gcacgtgtgt cgggtcgaac ctcgcttgct ggatgtggct tgactctatg
1611 gatgtcgcac tcgatactct ttcgcggtgc gaagggcttc cctctttccg cctgtccata ggccattcgc
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1751 gcccaaagcg gtggagactg aactcgcagc taaaaacact acgagcagtc cccccgcctc ggataccttt
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1891 gcaatagggg actaagacac ctattggcat aatggcggaa actcactcga ctatggcgag cggcgtcggc
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2031 cgcgcaaccg gctaagtaat tacgtcgacc gtgctgtcca aagggctgac ctttcgcccg tcactcgcgt
2101 tgcgttaatt acactcaatc gagtgagtaa tccgtggggt ccgaaatgtg aaatacgaag gccgagcata
2171 caacacacct taacactcgc ctattgttaa agtgtgtcct ttgtcgatac tggtactaat gcggttcgac
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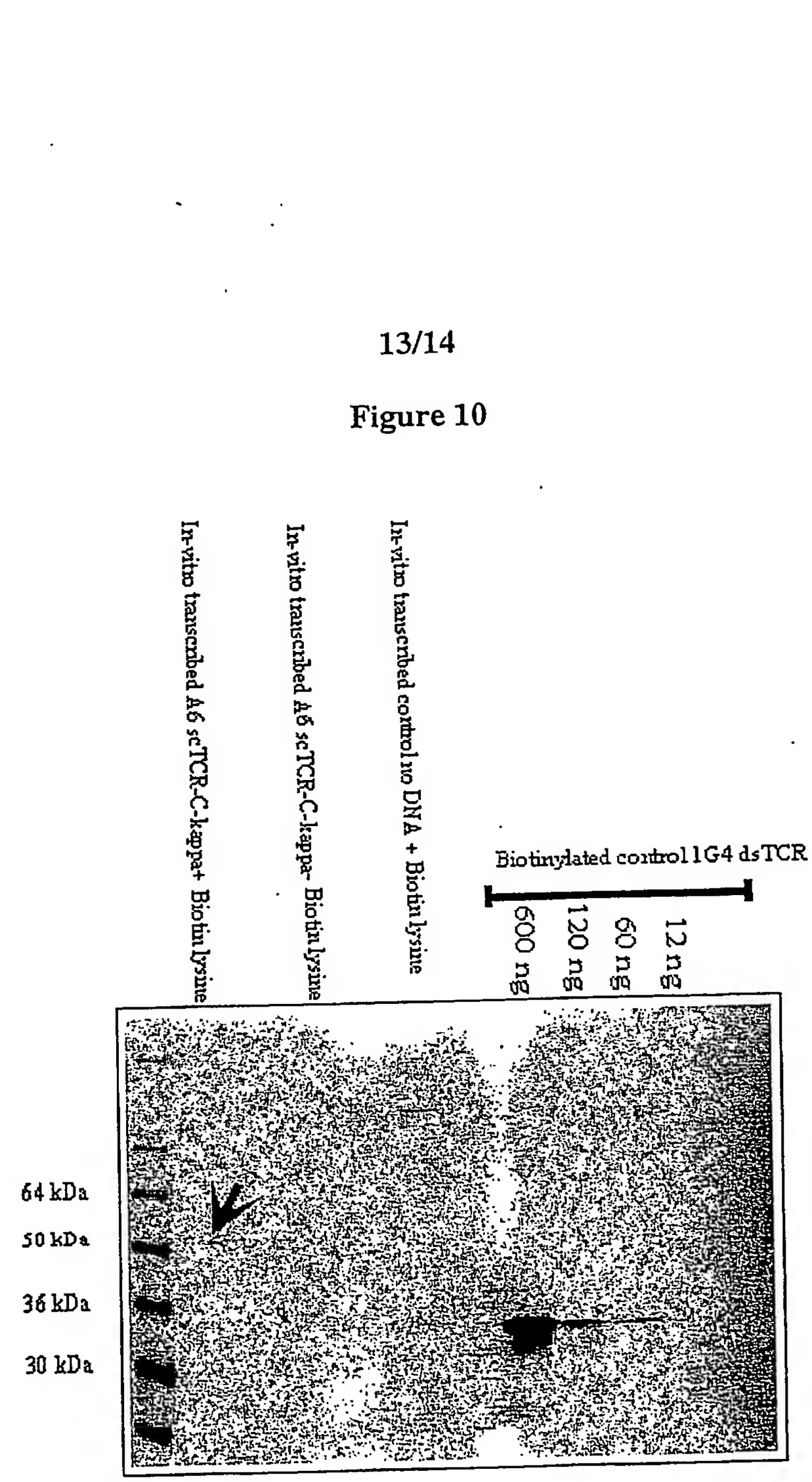
2521 gtgagagtca tgttagacga gactacggcg tatcaattcg gtcggggctg tgggcggttg tgggcgactg
2591 cgcgggactg cccgaacaga cgagggccgt aggcgaatgt ctgttcgaca ctggcagagg ccctcgacgt
2661 acacagtctc caaaagtggc agtagtggct ttgcgcgct
```

Figure 9

A6 scTCR-C-kappa cloned into pUC19-T7

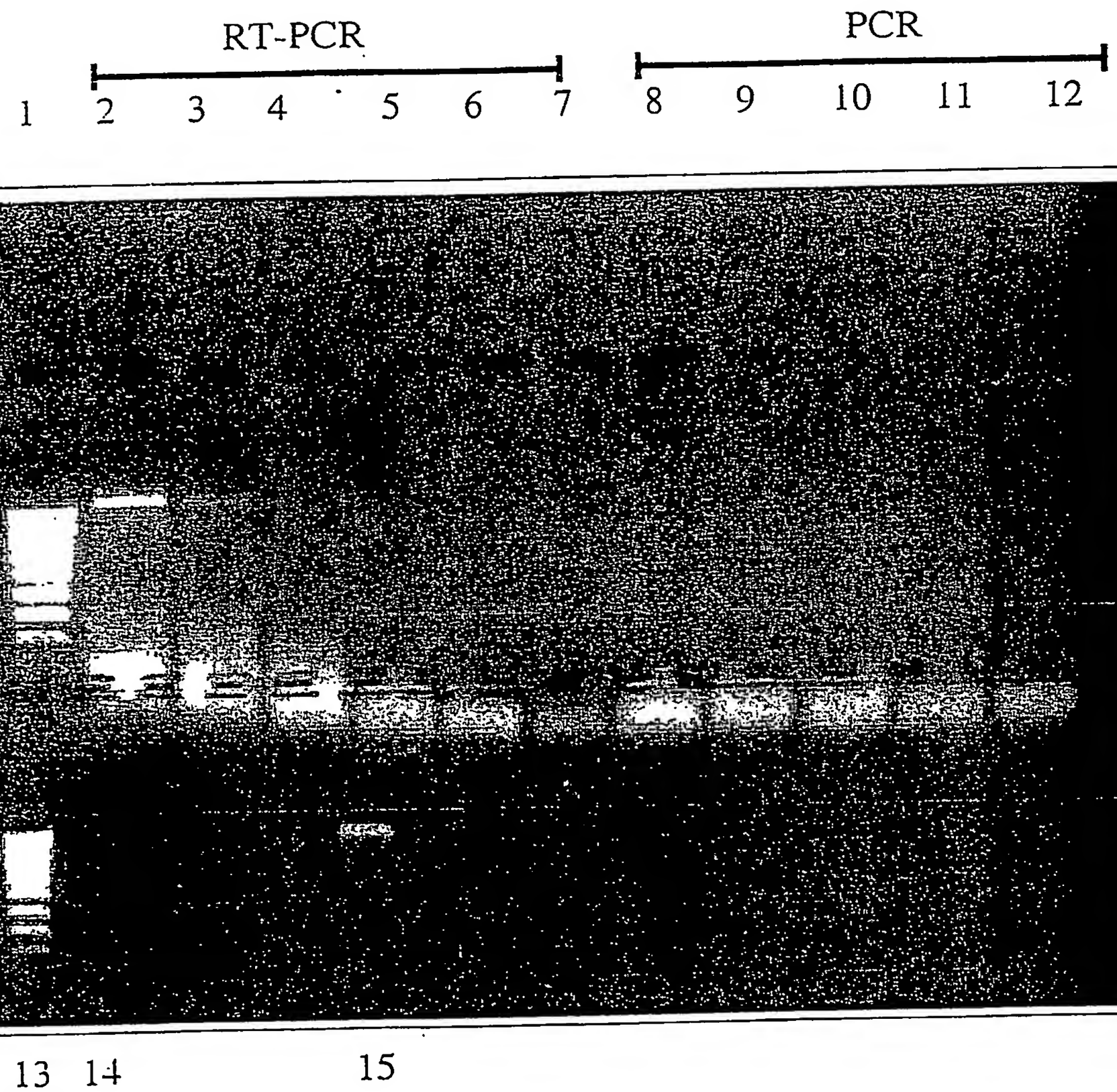
```
   1 ccatgggcca gaaggaagtg gagcagaact ctggacccct cagtgttcca
  51 gagggagcca ttgcctctct caactgcact tacagtgacc gaggttccca
 101 gtccttcttc tggtacagac aatattctgg gaaaagccct gagttgataa
 151 tgtccatata ctccaatggt gacaaagaag atggaaggtt tacagcacag
 201 ctcaataaag ccagccagta tgtttctctg ctcatcagag actcccagcc
 251 cagtgattca gccacctacc tctgtgccgt tacaactgac agctggggga
 301 aattgcagtt tggagcaggg acccaggttg tggtcaccgg tggaggcggt
 351 tcaggcggag gtggatccgg cggtggcggg tcgaacgctg gtgtcactca
 401 gaccccaaaa ttccaggtcc tgaagacagg acagagcatg acactgcagt
 451 gtgcccagga tatgaaccat gaatacatgt cctggtatcg acaagaccca
 501 ggcatggggc tgaggctgat tcattactca gttggtgctg gtatcactga
 551 ccaaggagaa gtccccaatg gctacaatgt ctccagatca accacagagg
 601 atttcccgct caggctgctg tcggctgctc cctcccagac atctgtgtac
 651 ttctgtgcca gcaggccggg actagcggga gggcgaccag agcagtactt
 701 cgggccgggc accaggctca cggtcacaga ggacctgaaa aacgtgttcc
 751 cacccgaggt cgctgtgttt gagccatcag aagcagagat ctcccacacc
 801 caaaaggcca cactggtgtg cctggccaca ggcttctacc ccgaccacgt
 851 ggagctgagc tggtgggtga atgggaagga ggtgcacagt ggggtcagca
 901 cagacccgca gccctcaag gagcagcccg ccctcaatga ctccagatac
 951 gctctgagca gccgcctgag ggtctcggcc accttctggc aggaccccg
1001 caaccacttc cgctgtcaag tccagttcta cgggctctcg gagaatgacg
1051 agtggaccca ggatagggcc aaacccgtca cccagatcgt cagcgccgag
1101 gcctggggta gagcagacgg tggaggcggt tcacttagca gcaccctgac
1151 gctgagcaaa gcagactacg agaaacacaa agtctacgcc tgcgaagtca
1201 cccatcaggg cctgagttcg cccgtcacaa agagcttcaa ccgcggagag
1251 tcataagaat tc
```

Figure 10



The A6 scTCR-C-Kappa protein is shown in the above western blot with an arrow.

Figure 11



Lane 1 Bioline 100bp DNA marker
Lane 2 A6scTCR-C-Kappa reaction selected againt HLA-A2 TAX beads
Lane 3 A6scTCR-C-Kappa reaction selected againt HLA-A2 TAX beads in the presence of 10 microgrammes of soluble A6scTCR
Lane 4 A6scTCR-C-Kappa reaction selected againt control beads
Lane 5 Control no DNA reaction selected against HLA-A2-TAX beads
Lane 6 Control no DNA reaction selected againt HLA-A2 TAX beads in the presence of 10 microgrammes of soluble A6scTCR
Lane 7 Control no DNA reaction selected againt control beads
Lanes 8-12 and lane 13 are as lanes 2-7 except no rerverse transcriptase was added just Roche high fidelity taq. These are the DNA contamination controls.
Lane 13 RT-PCR positive control.

PCT Application
GB0304636